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CANCER RESEARCH

A MONTHLY JOURNAL OF ARTICLES AND ABSTRACTS REPORTING CANCER RESEARCH

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Hormonal Imbalances in Tumorigenesis*

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We are now probably all more aware of "hormonal imbalances" than ever before. Investigators of problems in experimental endocrinology have been aware of *hormonal imbalances* or *balances* for many years. Now the terms are used more frequently because they are being considered in connection with the production and treatment of tumors and cancer. An environment in which both the applied and fundamental aspects of a problem are being investigated in the clinic and in the laboratory simultaneously accelerates the expansion of that particular field of investigation. It is of interest that two groups of investigators here in Chicago have contributed significantly to the understanding of hormonal interreactions. Moore and Price (27) first expanded the theory of hormonal interreactions involving the reproductive organs. Huggins and his associates (20, 21) were the first to modify hormonal balances to affect the treatment of prostatic cancer.

There are undoubtedly differences of interpretation of hormonal imbalances but that is natural, it is so hard to define a "normal" balance of hormones, particularly in connection with some systems of hormones. In fact, the hormonal balances change from day to day, year to year, or even moment to moment in some instances. Such changes are essential as the body develops, is adapted to different stresses and as it ages. The flexible state of many hormonal activities is worthy of contemplation, because, when appreciated, it implies that hormones are regulators or incitors of functions; that they do not initiate or need not initiate any new function or functions to which organized aggregates of living cells are not already subject. One cannot expect a hormone to do something that the body cannot do but it can modify or regulate functions for which the body has intrinsic and sometimes even nascent potentialities. Consider the thyroid, for example; energy can be used by the living body in the absence of the thyroid gland, but under normal conditions, the thyroid regulates some aspects of the level of metabolism. Although the adrenal glands function in the regulation of protein mobilization and carbohydrate storage and utilization, these functions are known to occur in adrenalectomized animals. Ovogenesis probably, and certainly some growth of ova and ovarian follicles occur in the absence of the pituitary gland, the source of gonadotrophic hormones that regulate the rate and the number of ova maturing and ovulating. The vaginal epithelium grows and heals and even cornifies in response to trauma in the castrated animal, and in the absence of known estrogenic hormones although the estrogens have profound effects on the growth of the vaginal epithelium.

Hormones should be looked upon, therefore, as substances regulating functions for which the body has inherent capabilities and usually some independent or autonomous basic level of response.

The apparent responses of the body to some hormones do not appear until relatively late. The prostate gland of the child is capable of responding to testosterone, but normally is small until puberty because the gonads are not stimulated adequately by, or do not respond to, the hypophyseal gonadotrophins. There is evidence that growth may occur in rats hypophysectomized when very young, whereas growth fails in older animals subsequent to hypophysectomy.

At least three variable elements must be considered in hormonal imbalance: 1. rate of production of hormones, 2. rate of destruction, elimination or utilization of hormone, and 3. the capacity of the "end organs" to respond. To this might be added a fourth possible variable, namely, the kind or quality of hormone produced (Table I). Each

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† The experiments undertaken by the writer were supported by grants from The Jane Coffin Childs Memorial Fund for Medical Research and The Anna Fuller Fund.

TABLE I: DISTURBANCES RESULTING IN HORMONAL IMBALANCES

1. Rate of production of hormones	Genetic influences Age—ontogenic stage Nutritional status Reciprocal glandular interactions Disease Other factors?
2. Rate of destruction or utilization of hormones	Genetic influences Age—ontogenic stage Nutritional status Specific inhibiting, augmenting or competing factors Disease
3. Capacity of end organs to respond	Genetic influences Age—ontogenic stage Nutritional status Inhibiting and augmenting hormones Duration and continuity of stimulus
4. Quality of hormone produced	Genetic influences Age—ontogenic stage Nutritional status? Disease?

of these variables is influenced by what might be termed (a) genetic or inherited influences that are characteristic of the strain, species or individual, (b) age or the stage of ontogenesis of the endocrine glands and end organs, and (c) nutritional status of the animal. All of these variables have been associated with the induction of specific imbalances in one or more, if not in all, instances. Additional influences may modify the three major elements that contribute to hormonal imbalances. Imbalances once induced may be temporary and may disappear or they may persist and incite irreversible changes in the end organs or associated structures. The entire problem of endocrine balances invites much further investigation.

First consideration will be given two classical experimental endocrine or hormonal imbalances involving the reproductive organs and then the hormonal pathogenesis of experimentally induced tumors of the anterior pituitary gland, testes, adrenal cortex and ovaries will be discussed. The humoral factors in the etiological background of some tumors of these organs have been studied most satisfactorily. Hormonal imbalances also are undoubtedly involved in cancer of the mammary glands in mice, in experimentally induced leukemia and possibly in cancer of the uterine cervix (11).

For the greater part, the exceptions to be noted later, the following investigations have been undertaken in our laboratories. Dr. C. W. Hooker, Dr. C. A. Pfeiffer, and Dr. Min Hsin Li have participated in many aspects of these problems or have conducted independent investigations on different aspects of the problems. The writer acknowledges their contributions.

An example of an endocrine imbalance that produces permanent physiological and morphological disturbances was described by Pfeiffer 11 years ago (27) (Fig. 1). Testes from newborn male rats were transplanted into their litter-mate sisters. The influence of the graft affected the young host during the first weeks of postnatal life because the transplanted testes could be removed a few weeks later and the animals were comparable to hosts in which the grafts remained intact throughout life. The testicular graft, by its endocrine action, changed the female host's pituitary into a physiologically male-type gland and it remained so throughout life. It had been known for years that ovaries grafted into intact male rats or mice do not show ovulation and corpora lutea rarely form. The male hosts' pituitaries produced a qualitatively different response in the ovary. The females that had grafts of testes during the first few weeks of life, as mentioned, had ovaries that likewise failed to form corpora lutea and did not contain ovulatory follicles. The hosts showed prolonged or constant estrus. They were sterile.

The injection of luteinizing hormone (L.H.) of the pituitary gland or from pregnant mare's serum (PMS) resulted in ovulation and corpora lutea then formed (28, 29). Thus in rats and mice grafted testes modify the functional qualities of the hypophysis; the altered hypophysis modifies ovarian function, and this in turn modifies the response of the end organs concerned, the mammary glands, uterus and vagina. A hormonal imbalance is instigated that, present during a short time, gives permanent functional abnormalities—a permanent hormonal imbalance. Exposure to androgens during early postnatal life induces similar physiological abnormalities (33).

Another interesting type of humoral imbalance has been studied in parabiotic rats (34). When an intact female rat is placed in parabiosis with a castrated female or male rat its ovaries become greatly enlarged, its genital tissues are excessively stimulated and its pituitary gland enlarges (Fig. 2). The gonadotrophic hormones of the castrated parabiont increase greatly and stimulate the ovaries of the intact parabiont; they pass from one animal to another. The ovarian hormones do not attain threshold levels in the castrated member so its uterus remains atrophic, and its pituitary gland remains hyperactive. The high levels of ovarian

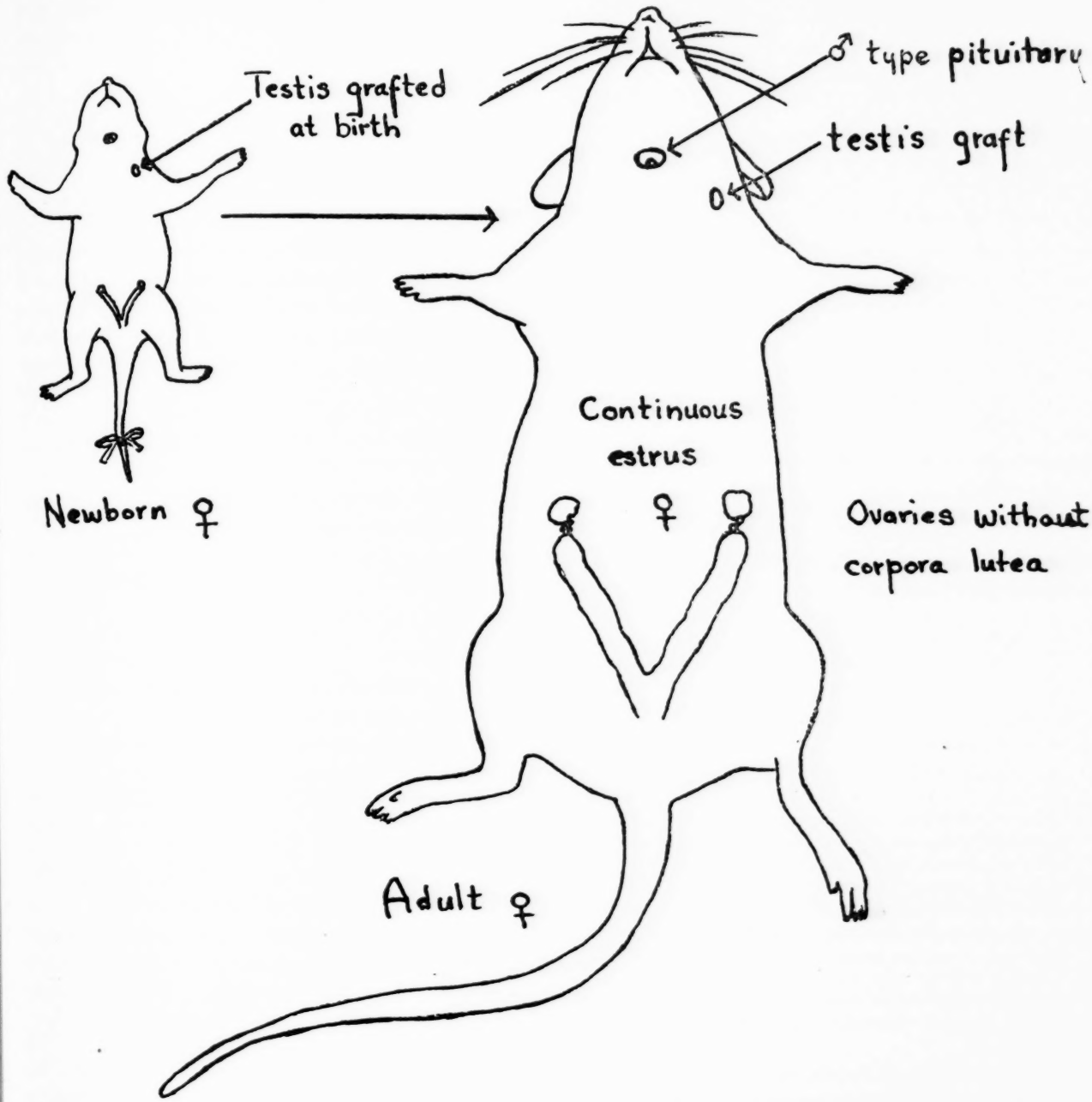


FIG. 1.—Schematic presentation of influence of immature testis on newborn female rat. Testis transplanted at birth modifies female host's pituitary gland irreversibly so that it functions much as would hypophysis of a male. Small but continuous production of ovarian hormones, primarily estrogenic, stimulates uterus excessively (Based on data from Pfeiffer [27]).

hormone in the intact animal, however, cause pituitary hyperplasia and hypertrophy. These examples of abnormal or atypical physiological and morphological changes are cited to show that endocrine disturbances readily attainable by "physiological levels" of hormones produce profound changes.

Pituitary tumors occur in estrogen-treated mice of some strains; in our experience they occur most frequently in estrogen-treated mice of the C57 black strain or in hybrids involving this strain (12). Estrogen-treated mice of at least 6 other strains rarely or never acquire such tumors, and they are extremely infrequent in control mice. The only untreated mouse with a pituitary tumor that the writer has observed also had bilateral granulosa

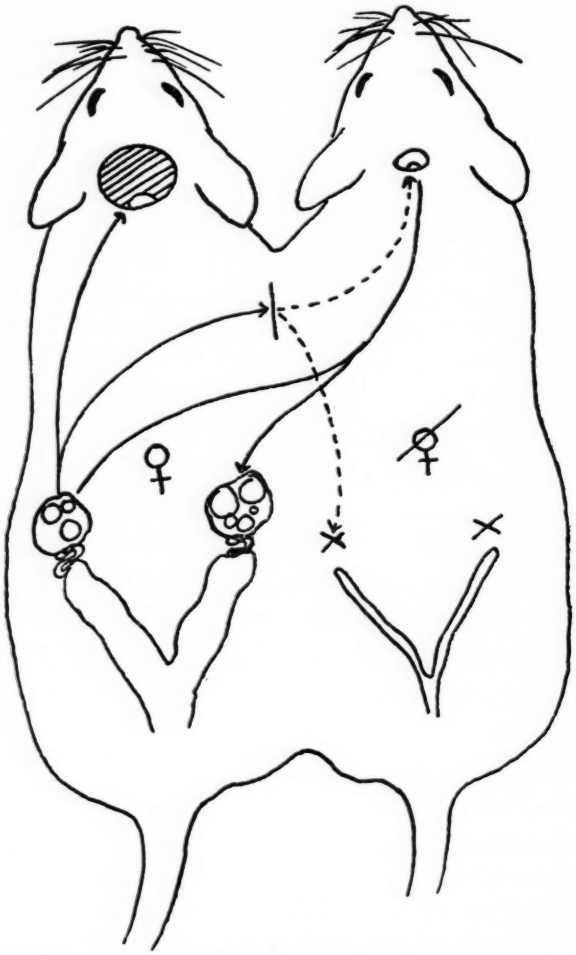


FIG. 2.—Schema of endocrine imbalances induced by parabiotic union of castrate and intact female rat. Inability of ovarian hormones to attain threshold levels in castrated member of pair results in (a) atrophy of its genital organs, and (b) excessive production and liberation of gonadotrophic hormone, primarily follicle stimulating hormone. Excessive gonadotrophin attains threshold levels in intact member and results in excessive ovarian stimulation and coincident stimulation of accessory genital tissues and of pituitary hyperplasia (Witschi [34]).

cell tumors (8). The proper endocrine and genetic influences must co-exist for tumors of the pituitary to develop.

The hypophyseal tumors that develop in estrogen-treated mice are chromophobe adenomas. They arise in localized areas of hyperplastic glands and may attain large size; up to 300 mgms., a mass almost as large as a normal mouse's brain (10). The tumors are usually fleshy and pink, are separated from the brain by the meninges and are not invasive. A few tumors contain large blood filled "cysts" and a few more contained one or more small cysts.

The tumors usually consisted of enlarged chromophobic cells arranged in cords. The cells contain large Golgi bodies and look like actively secreting cells. The tumors, however, contain little gonadotrophic hormone as revealed by bioassay in hypophysectomized mice (17). The low content of hormone in the hypertrophied glands is reminiscent of the iodine content of the thyroid of thiourea-treated animals. Some tumors contain dilated sinusoids or hemorrhagic areas. They grow, once they have started, after the discontinuance of estrogenic treatment; they grow subsequent to transplantation in other mice of the same strain if the hosts are given estrogen. In our laboratory they have not grown in mice that have not received estrogen and are thus not completely autonomous. The simultaneous injection of androgen will reduce the incidence of such tumors.

The tumors usually appear in animals that have received estrogen for 350 days or more and at an earlier age in males than in females. For purposes of classification any enlargement of the pituitary gland in excess of 12 mgm. has been considered a tumor. Some of these, however, are markedly hypertrophied glands, although they exceed by at least 6 times the size of a normal female mouse's pituitary. However, even small glands contain localized adenotomous nodules. Many of the tumors may exceed 50 mgm. in weight (Fig. 3).

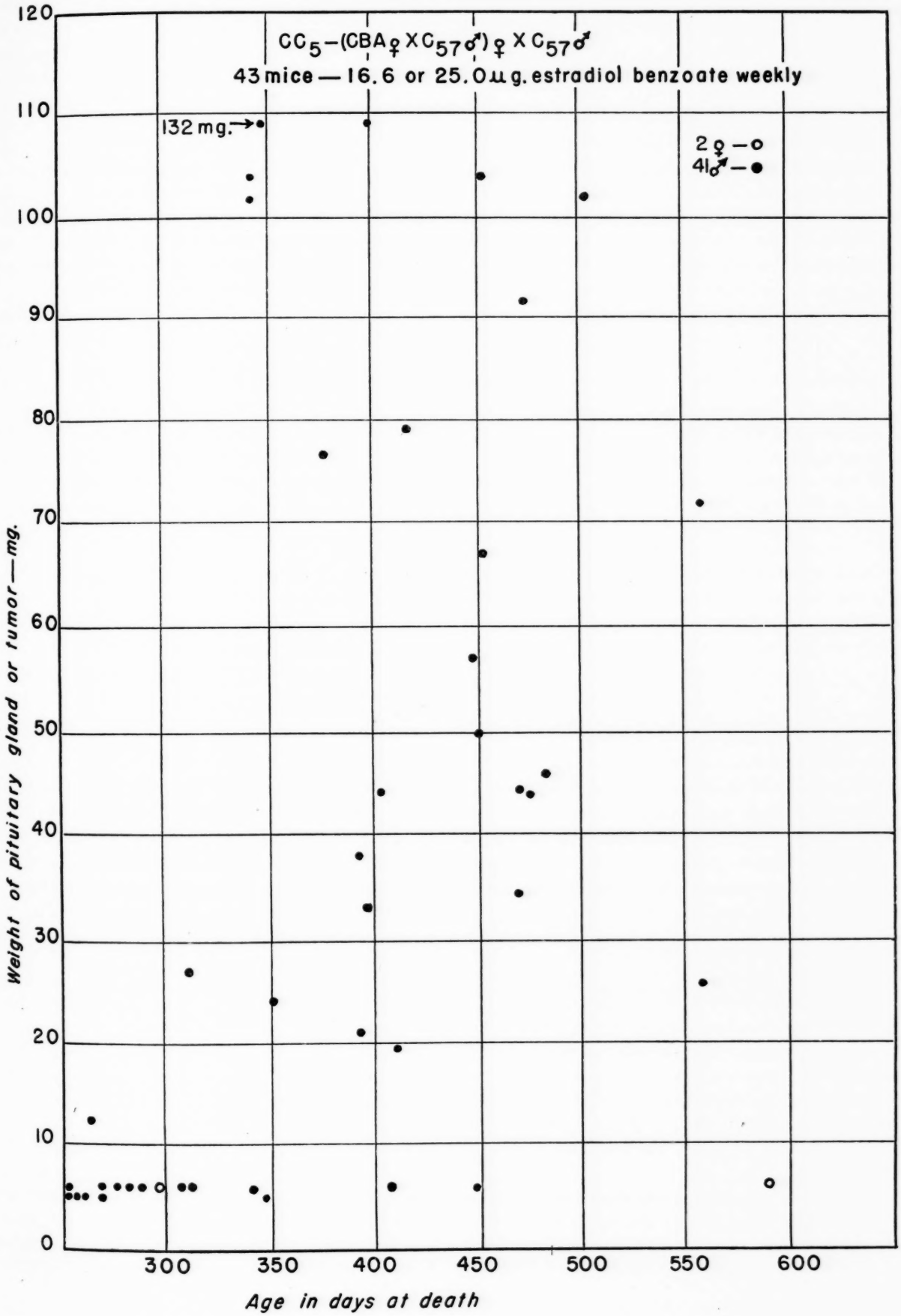
The tendency for estrogen-treated mice of the C57 strain to acquire hypophyseal tumors is transmitted genetically, by both the males and the females (10). The incidence of hypophyseal tumors in estrogen-treated backcrossed mice is roughly

TABLE II: THE INCIDENCE OF PITUITARY TUMORS AMONG ESTROGEN-TREATED HYBRID AND BACKCROSS MICE

Group	Origin of group	Sex	Number of mice	With pituitary tumors		Age of tumor		Average age at death nontumor animals days
				Number	Per cent	Average days	Range days	
CC_1*	C57 ♀ × CBA ♂	♂	23	19	83	513	326–671	320
CC_2*	CBA ♀ × C57 ♂	♂	24	18	75	480	380–543	360
CC_3	(CBA ♀ × C57 ♂) ♀ × CBA ♂	♂	38	8	21	517	390–592	372
CC_4	(C57 ♀ × CBA ♂) ♀ × CBA ♂	♂	34	7	21	525	324–647	429
CC_5	(CBA ♀ × C57 ♂) ♀ × C57 ♂	♂	41	26	63	419	263–577	300

* Data published in Cancer Research, 1:345. 1941.

FIG. 3.—Scatter chart of weights of pituitary tumors from mice that had received estradiol benzoate beginning at ages 21 to 45 days and continuously thereafter until death.


CC5—(CBA♀ X C57♂)♀ X C57♂
43 mice — 16.6 or 25.0 µg. estradiol benzoate weekly
132 mg.
2♀ — o
41♂ — ●
Weight of pituitary gland or tumor—mg.
120
110
100
90
80
70
60
50
40
30
20
10
0
300
350
400
450
500
550
600
Age in days at death

proportional to the amount of C57 chromatin that the animals carry (Table II). From 75 to 83 per cent of the male mice of the F_1 generation acquired chromophobe adenomas of the pituitary subsequent to estrogen treatment at an average age of 480 to 513 days. Only 21 per cent of the animals derived by backcrossing the first generation hybrids to the CBA strain acquired pituitary tumors, whereas 63 per cent of the backcrosses to the C57 strain had chromophobe adenomas at death. The incidence of tumors in the latter group would be higher if corrections were made for age; the average survival of mice in this group was 419 days.

In summary it may be said that (a) chromophobic adenomas of the pituitary glands of mice develop when the proper animals received estrogenic hormones, (b) the tendency for the tumors is transmitted by both males and females to their hybrid offspring (Fig. 4), (c) the tumors produce little hormone of any type but do apparently produce some hormone, (d) simultaneous administration of androgens reduces the incidence of these tumors, (e) the tumors appear at an earlier age in estrogen-treated males than in females, (f) the tumors are transplantable in estrogen-treated mice, and (g) once the tumors become established they develop in the absence of the environment necessary for their inception or origin.

Do such observations prove that chromophobe adenomas that occur rarely in untreated mice and in man are attributable to an abnormal presence of estrogens? It would be rash to be dogmatic on this point. Nevertheless, there is no reason to believe that mice of one strain differ from mice of another strain any more than one person differs from another. One person but not another might acquire a pituitary tumor if subjected to prolonged treatment with estrogen. In man the pituitary gland of the female is larger than that in the male, it enlarges during pregnancy and its gonadotrophic function decreases subsequent to estrogen treatment. In man the pituitary gland thus shows responses that resemble those of the mouse. Estrogens act on the pituitary gland much as thiourea acts on the thyroid in that they prevent or reduce the secretion of some, but not all, of the hormones that the hypophysis normally produces. Under such conditions the gland hypertrophies and in the pituitary adenomas form in animals of some strains.

Interstitial-cell tumors of the testis developed in estrogen-treated mice of two strains in our laboratory, the A and JK strains, but very rarely in mice of other strains when they are treated similarly (14, 19) (Fig. 5). There is no apparent tendency for untreated mice of any one strain to acquire interstitial cell tumors because the few "spontaneous" tumors that have been observed have occurred in mice of several strains (13). Interstitial cell tumors in estrogen-treated mice may appear in one testis or in both simultaneously. The testes bearing tumors are usually considerably enlarged, or even greatly enlarged, and yellowish or yellowish-brown in color. Metastatic growths are noted frequently in the iliac and in the perirenal nodes, less frequently in the lungs. Metastases occur only when the tumors become quite large.

The tumors produce androgen. The seminal vesicles of the host are often large and filled with secretion although the animals bearing them have received estrogen until the time of necropsy (19). In estrogen-treated mice without testicular tumors the seminal vescicles are usually small and atrophic as would be expected because the testes are small and atrophic and produce small or negligible amounts of androgen. (Estrogen does induce an increase in the fibromuscular tissue of the seminal vesicles and a squamous metaplasia of the epithelium of the coagulating gland and sometimes of the seminal vesicles.)

The tumors consist, for the greater part, of large cells not unlike normal Leydig cells (Fig. 6). These cells replace, with progressive development of the tumors, all of the normal constituents of the testes and extend through the tunica albuginea. In small areas of the tumors, or making up large proportions of the growths of a few tumors, are groups of small cells. Mitotic figures occur more frequently in the smaller and more hyperchromatic cells. Hooker and Pfeiffer (19) have studied this problem extensively, and consider that these cells are the primary hyperplastic elements of the tumor; that they differentiate in some tumors into typical large Leydig cells such as are found in most of the tumors.

The testes of estrogen-treated mice of those strains not susceptible to testicular tumors show marked atrophy of the seminiferous tubules; much as would occur subsequent to hypophysectomy (9). The Leydig cells are atrophic; the intertubular spaces are small and contain small groups of macrophages.

The damage to the seminiferous tubules in the estrogen-treated mice of the A strain is not as great as in mice of most strains (9). Estrogen has a qualitatively different effect upon the pituitaries of mice of this strain from that upon mice of strains in which testicular tumors do not appear. The inter-

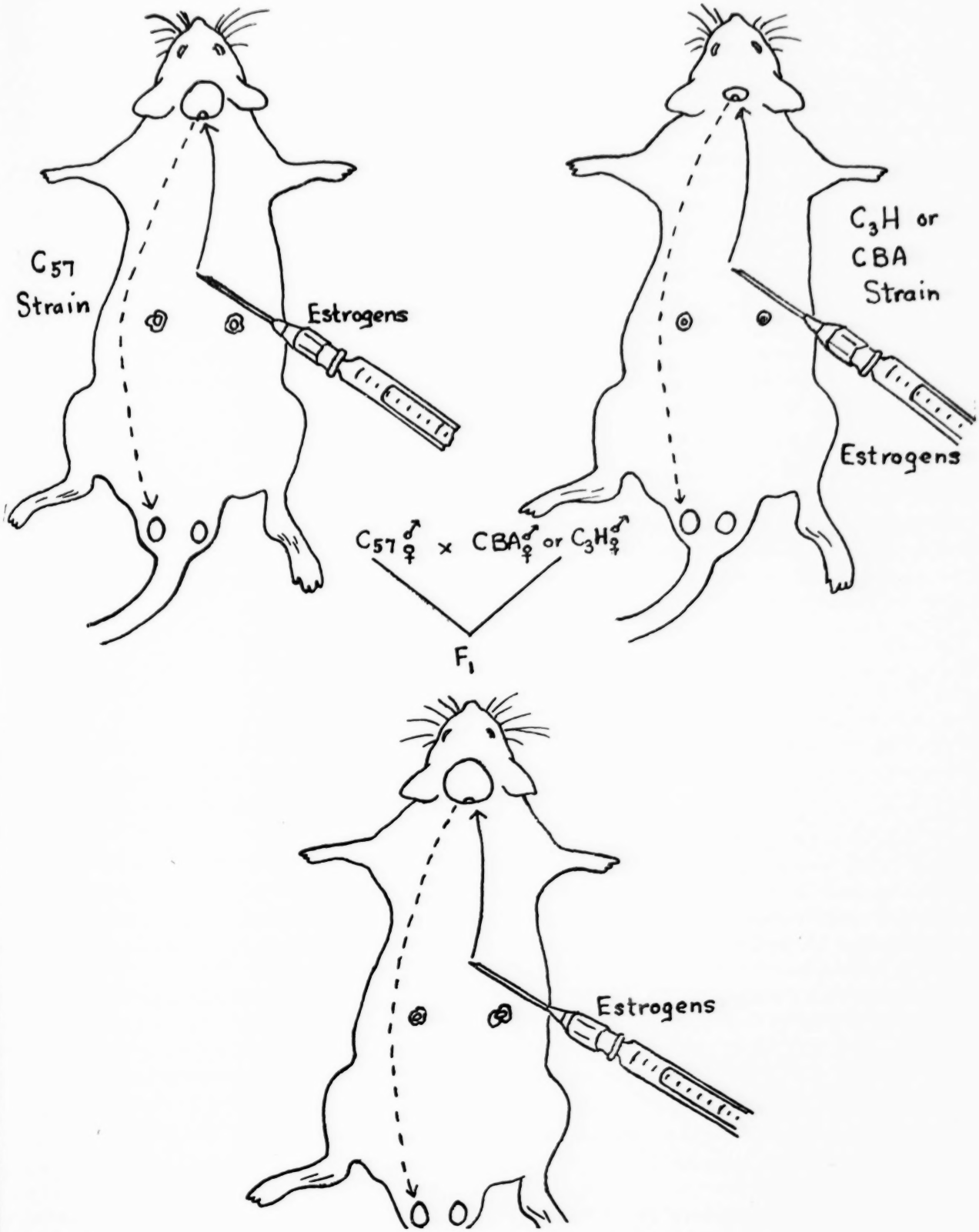


FIG. 4.—Interpretative schema of hormonal and genetic influences in experimentally induced pituitary tumors. The tendency for chromophobe adenomas of the pituitary is transmitted by both male and female mice of the C57 strain to their F_1 offspring.

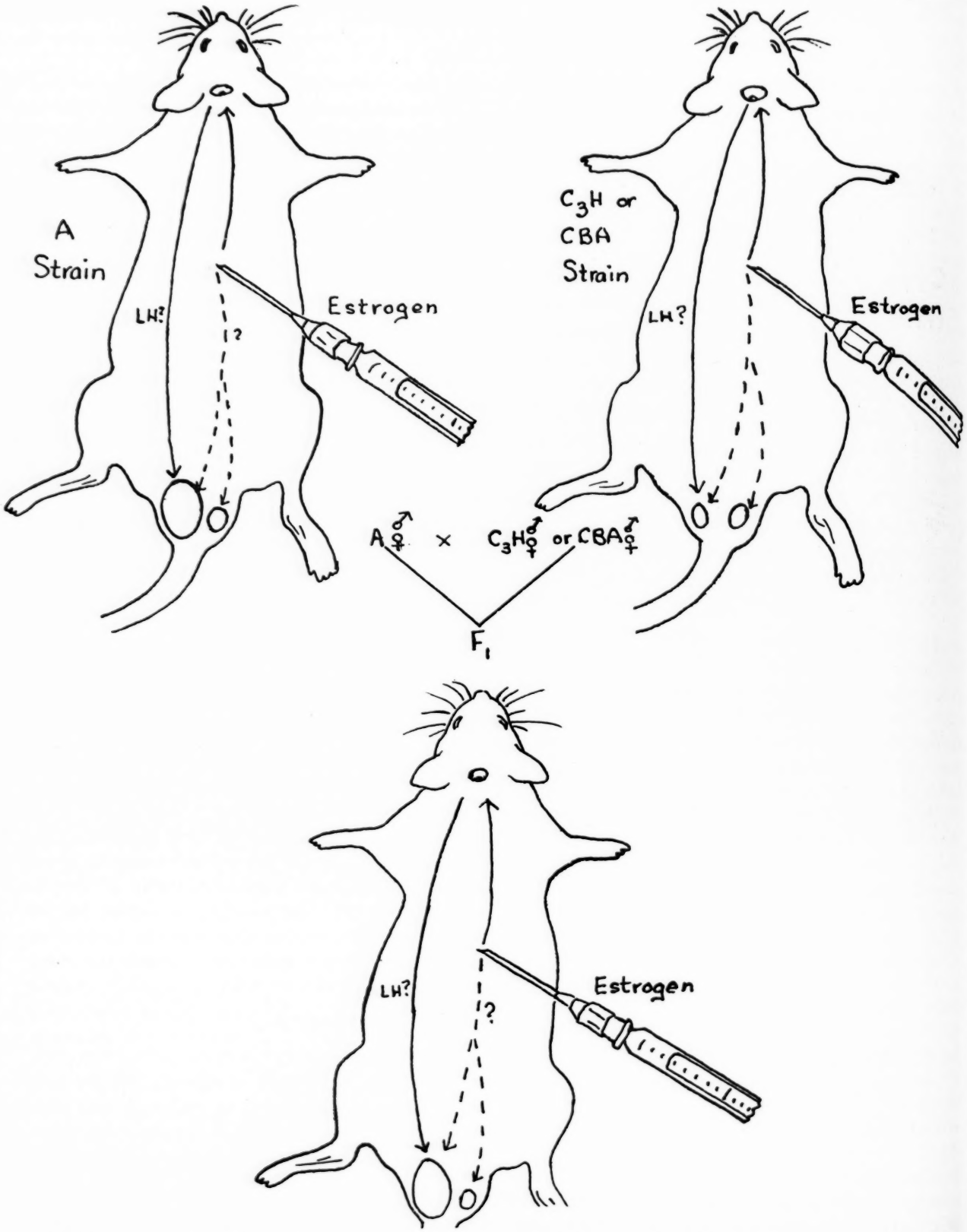


FIG. 5.—Interpretative schema of hormonal and genetic influences in experimentally induced tumors of testicular interstitial cells. It is assumed that estrogens cause greater production of gonadotrophin (luteinizing hormone) in animals of the A strain. (The possibility still exists, however, that the end organ, namely the testes, of the A strain may differ from those of other strains.) The tendency for these tumors is transmitted by both male and female mice of the A strain.

stitial cells of the testes of estrogen-treated mice first hypertrophy, are then depleted and are replaced by brown cells—macrophages. Subsequently local foci of hyperplasia of the interstitial elements occur, forming small, nodular tumors and finally the larger tumors. The sequences of progressive changes in interstitial elements of estrogen-treated mice of the A strain have been described in detail (19).

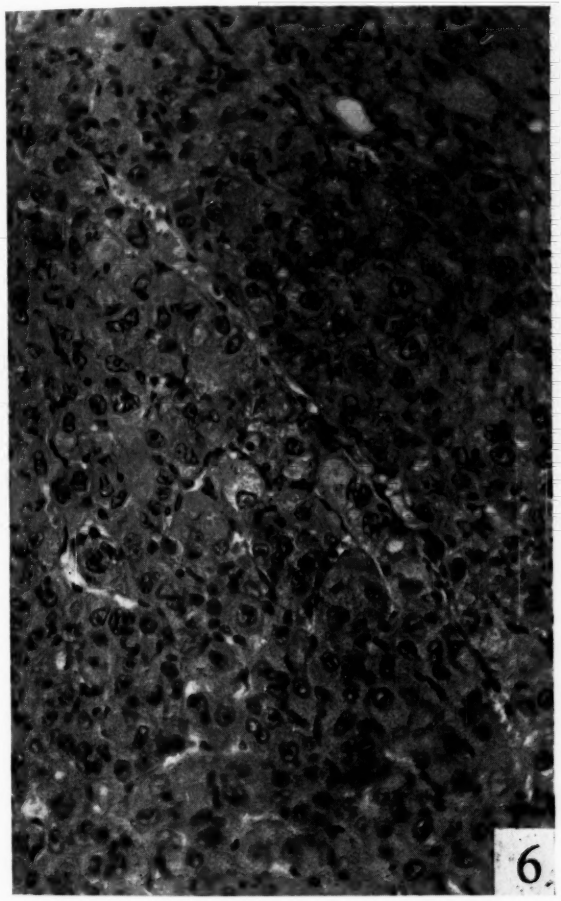


FIG. 6.—Photomicrograph of interstitial cell tumors of testis of a mouse of the A strain that had received 250 μgm. of stilbestrol weekly for 34 weeks, beginning at 36 days of age. This tumor consists of large and well differentiated cells. Similar tumors have appeared in mice of the A strain given equilin benzoate, estrone or estradiol and several esters of these substances, stilbestrol and tryphenylethylene. Mag. × 220.

Not only is an altered balance of hormones necessary for the genesis of testicular tumors but it is also necessary for their growth subsequent to transplantation in a new host. The interstitial cell tumors of the testis grow when they are transplanted into mice of the same strain provided the hosts receive estrogen (15). They have not grown in untreated males and few will grow in untreated females. Fragments of the tissue may persist in the subcutaneous tissues for several months and can be found only upon the closest inspection. Histological study of these remnants reveal tissues that are scarcely identifiable and certainly not similar to that of the original grafts. If, however, after the transplant has remained dormant for a period of several months estrogen is administered, the grafts begin to grow. In fact, they grow as rapidly as does a second graft implanted into the same mouse at the time the administration of estrogen is begun. After the tumors have started to grow estrogen treatment may be discontinued and the tumors grow progressively (15).

If male or female mice of the A strain are hybridized with animals of strains not susceptible to testicular tumors, such tumors occur in the estrogen-treated hybrids. The tendency is transmitted by both males and females (Fig. 5).

If animals of the A strain are mated with animals of the C57 strain, the strain in which pituitary tumors develop, then both the pituitary tumors and testicular tumors may occur in the same animal (Fig. 7). They are not mutually incompatible.

How can these observations be explained? Do the estrogenic hormones act directly upon the testes as a carcinogenic hydrocarbon might act on skin, or is the tumorigenic action mediated indirectly? It is probable that the effects upon the testis are mediated through the pituitary (Figs. 5, 7). It has been known for several years that the injection of estrogenic hormone increases the production of luteinizing hormone (L.H.) in acute experiments—experiments of short duration (5, 22). It has been demonstrated that L.H. stimulates the interstitial cells of the testis and is identical to interstitial cell stimulating hormone. The interstitial cell tumors are assumed to arise in an environment of increased or continuous stimulation by intrinsic L.H., a stimulation induced in turn by prolonged exposure to estrogenic hormones, the latter, in these experiments of extrinsic origin. If this assumption is true it could be tested by subjecting susceptible animals to luteinizing hormone for prolonged periods. Such experiments have been undertaken but they are difficult to evaluate because the antigenic nature of most gonadotrophic preparations that have been used leads to the production of antihormones so that no more than indications of the correctness of this hypothesis can be obtained (30). The only known experimental means of inducing an animal to increase the production of its own luteinizing hormone is by the injection of estrogenic hormone and this quality may be particularly

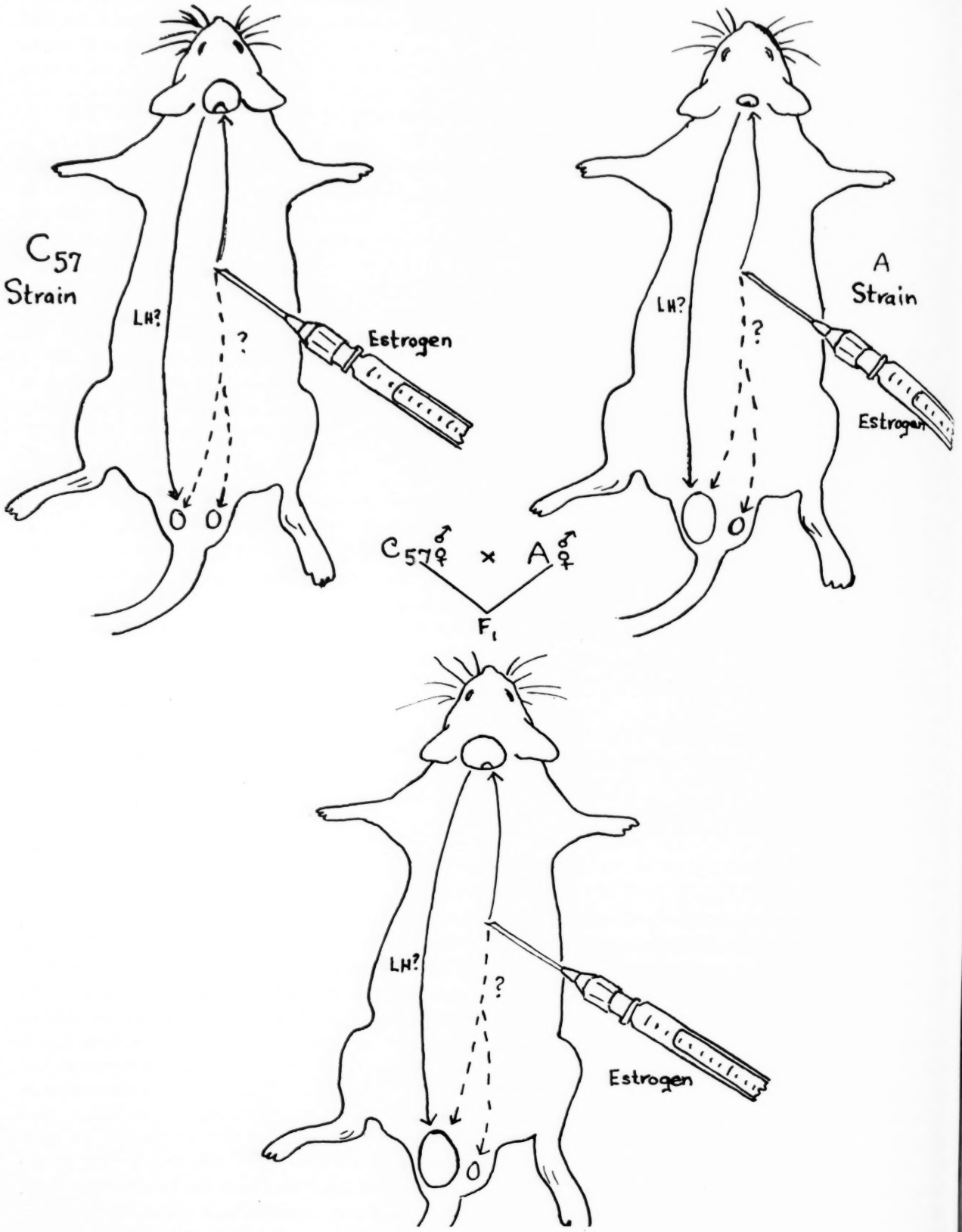


FIG. 7.—Interpretative schema of hormonal and genetic influences in hybrids obtained by crossing mice of the A and C57 strains.

pronounced in mice of the A strain. An endocrine environment of increased and prolonged exposure to L.H. is assumed to be responsible for testicular interstitial cell tumors in estrogen-treated mice of some strains. The possibility exists, however, that estrogens act directly upon the testis in inciting interstitial cell tumors.

Interstitial cell tumors rarely occur in man, but more frequently in horses and in dogs. It is of interest that the stallion excretes large amounts of estrogen.

Tumors of the adrenal cortex occur under conditions indicative of the etiological role of an endocrine imbalance. They were first described in castrated mice by Woolley (35), and in castrated guinea pigs by Spiegel (32). Mice castrated at birth, or in our experience up to two months of age (2), acquired, after many months, adrenal hyperplasias and, in mice of some strains, adrenal tumors. Dr. Woolley and his associates have shown that these tumors are prevented by the injection of gonadal hormones (38). In the absence of gonadal hormone the internal environment is proper for adrenal hyperplasia, and in some strains for the development of malignant growth in the adrenal cortex (Fig. 7). One might recall here the intimacy of the adrenal and gonadal tissues embryologically, their histogenic similarities and the chemical similarities of their secretions. It has been shown that some pituitary extracts induce the adrenals to produce the hormones that have androgenic activity (35). Although increases in adrenocorticotrophic hormone subsequent to castration have not been demonstrated there may be more than one hypophyseal adrenocorticotrophic substance. All that can be said for certain is that adrenal tumors occur in suitable mice in the absence of the usual source of gonadal hormones.

The adrenal cortical tumors differ histologically; some consist predominantly of small, hyperchromatic cells and others of cells with a more abundant cytoplasm that resemble the cells in the zona fasciculata of the normal gland. The tumors may become larger than the normal kidney, and metastatic growths have arisen from them (36, 37). Tumors of the adrenal cortex under such circum-

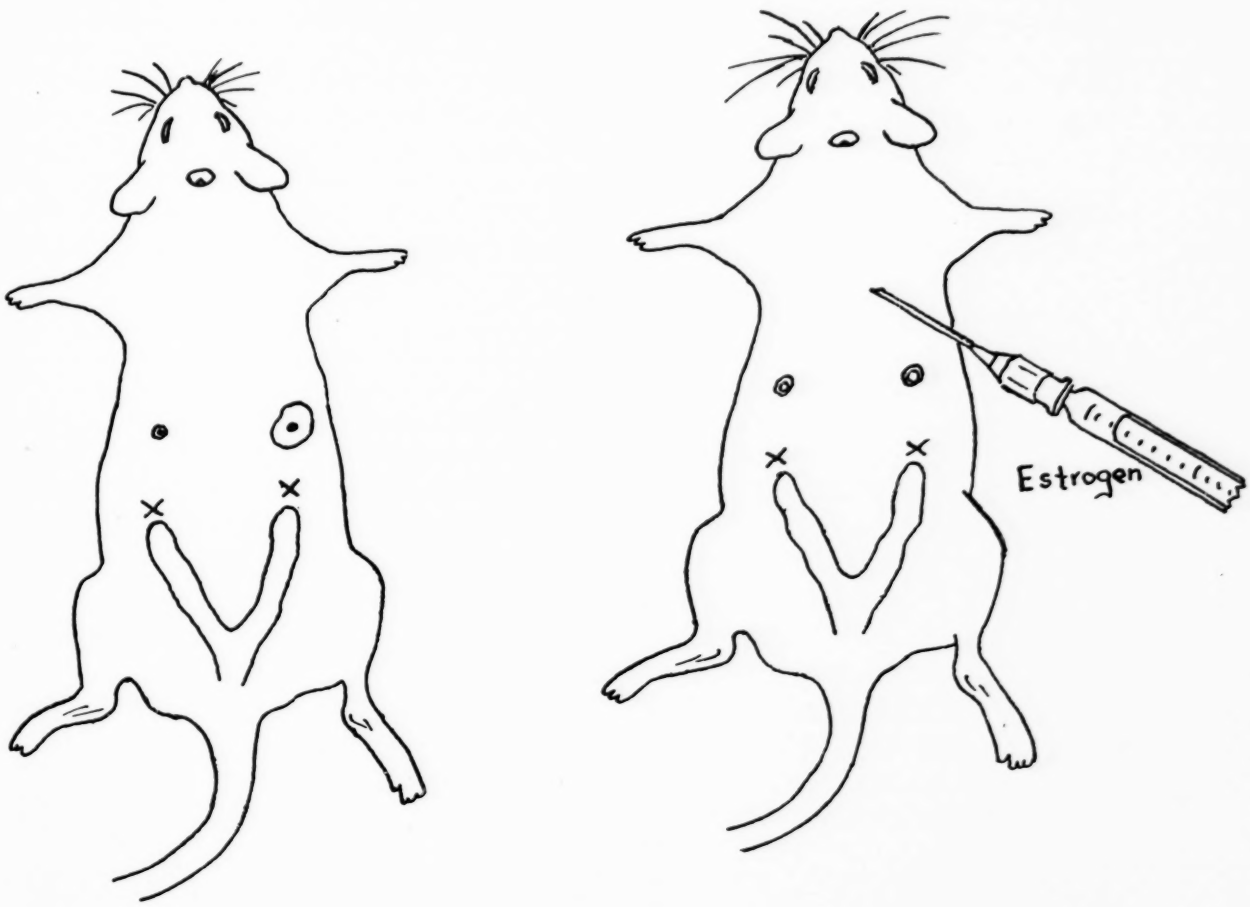


FIG. 8.—Schema of hormonal influences in the genesis of tumors of adrenal cortex (Wooley [35, 38]).

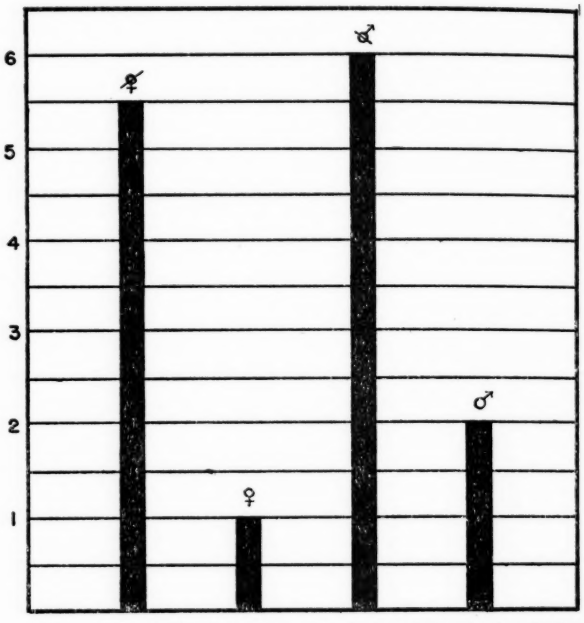
stances have grown subsequent to transplantation into other mice of the same strain.

The adrenal tumors in some animals produce estrogenic hormones. The mammary glands of the host, the vagina and uterus are stimulated (35). The tumors tend to replace the humoral deficiencies of the castrated host. Castrated animals with adrenal cortical tumors excrete in their urine and feces approximately four times as much estrogenic substance as do animals with intact ovaries (2). Woolley and his associates have found that adrenal cortical tumors in mice of some strains produced predominantly androgenic hormones, as judged by the effects upon accessory genital organs and other tissues of the host (37). Mice of some strains acquire adrenal cortical tumors more frequently than others subsequent to castration and the influences of genetic factors are being studied. A higher incidence of adrenal cortical tumors occurs in hybrid mice than in either of the parental stocks (31).

A marked hypertrophy of the adrenal gland also occurs in some mice receiving estrogenic hormones (17). The adrenal glands are enlarged 2 or 3 times, the cortex is disorganized, extends through the capsule and largely replaces the medulla. Excessive and continuous estrogen in the environment also is associated with abnormal proliferative changes of the adrenal glands.

Ovarian tumors have been associated recently with circumstances indicating the influence of hormonal imbalances on their origin. It has been known for many years that the pituitary glands of castrated male and female rats contain from three to six times as much gonadotrophic activity as do the pituitaries of the intact rats (3, 4) (Fig. 9). In man prolan A increases subsequent to the menopause—a well known fact. The removal of the gonads increases the production of gonadotrophin, especially of follicle stimulating hormone (F.S.H.). In fact, prolan A (the gonadotrophic substance in urine of post-menopausal women) has almost a specific follicle stimulating effect. From such data and from other experiments Moore and Price (26) based their theory of reciprocal gonad-hypophyseal relationship.

It has also been known for many years that estrogenic hormones administered intraperitoneally are less effective than when administered subcutaneously. Zondek (39) found that hepatic tissue of rodents could destroy estrogens *in vitro*. Others have found that, in rats, ovarian grafts placed in the mesentary do not stimulate the uterus and vagina of castrated hosts whereas the same ovaries removed and retransplanted subcutaneously induced vaginal cornification (18). Two years ago Biskind and Biskind (1) observed tumorous growths in intrasplenic grafts of ovarian tissue in three castrated rats and this problem has been studied extensively in mice (23, 24).



Levels of gonadotropins in intact and gonadectomized animals as determined by bioassays of pituitary tissue.
(Based on data of Engle, 1929; Evans and Simpson, 1929; and others.)

FIG. 9

Many tumors of ovarian tissue—granulosa cell tumors and luteomas—have developed in intrasplenic transplants of ovaries in castrated male and female mice of all of the five strains that have been studied (24). In several instances these tumors metastasized to the liver and they have grown, subsequent to transplantation, in untreated hosts of the strain of origin in almost all instances in which attempts have been made to transplant them. They attain large size, over two centimeters in diameter, when they are permitted to grow (24). Many of the tumors produce estrogenic hormone because the uteri and mammary glands of their hosts are well developed and they show prolonged periods of vaginal epithelial cornification.

The tumors arising in the castrated male mice have been predominantly granulosa-cell tumors. The cells are small, arranged in follicular or trabecular patterns and only in a few instances have they shown a tendency to undergo luteinization. They are similar to the rare spontaneous granulosa cell tumors observed in mice and to the ovarian tumors that develop in x-rayed mice (6, 7). On

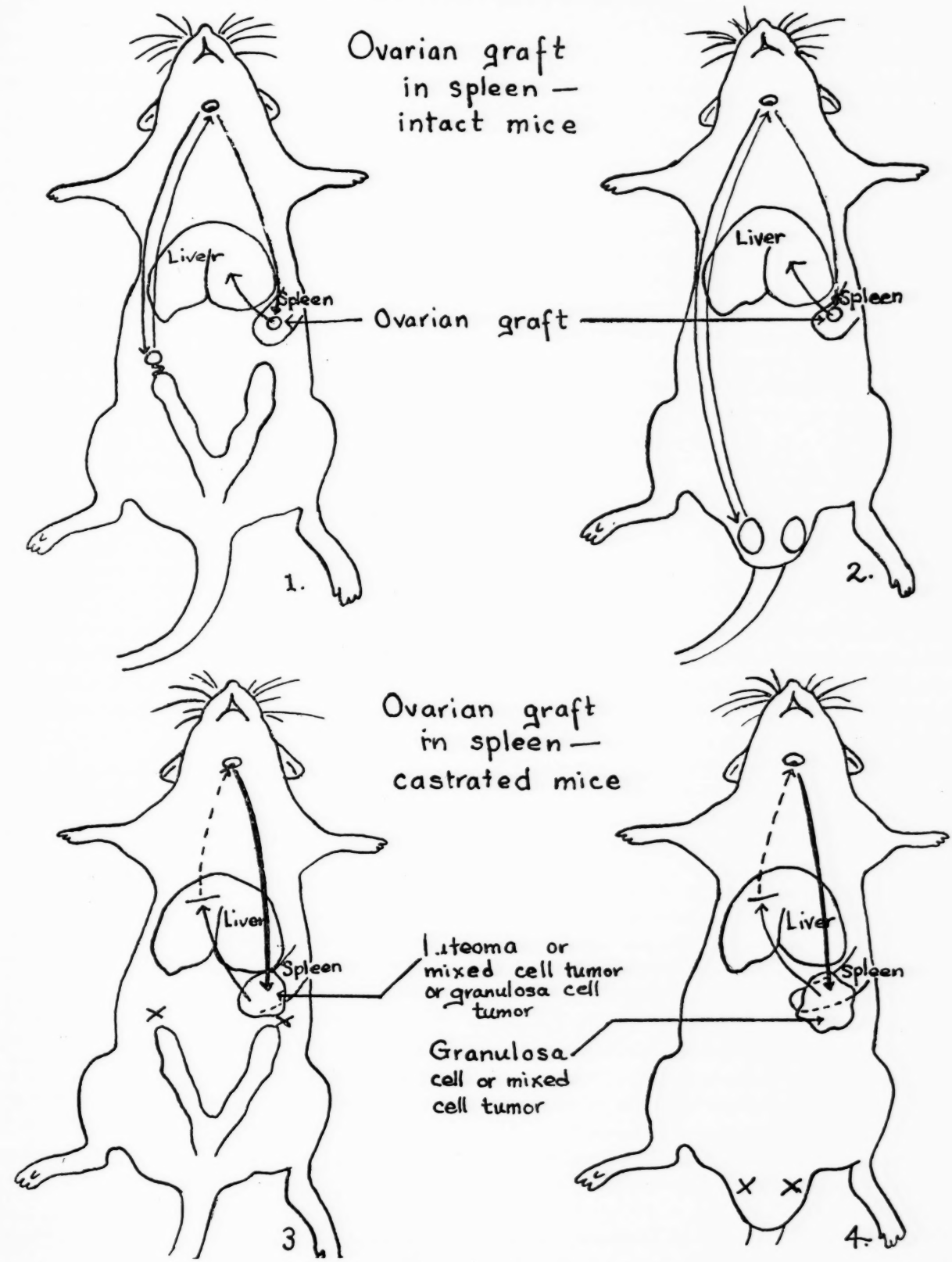


FIG. 10.—1 and 2: Interpretative schema of hormonal and metabolic influences on the genesis of granulosa cell tumors and luteomas in intrasplenic ovarian grafts. 3 and 4: In unilaterally gonadectomized males and females no tumors appear. In castrated males and females tumors appear.

the other hand, many of the tumors arising in the ovarian transplants in castrated female mice are either mixed granulosa-cell tumors and luteomas or luteomas. There is a sex difference in the predominant type of tumor arising under such conditions (23).

Intratesticular ovarian grafts in castrated mice and intrasplenic ovarian grafts into intact or unilaterally castrated mice failed to become tumorous (Fig. 10). Only one of a large series of subcutaneous ovarian grafts in castrated hosts became tumorous. Intrasplenic grafts with vascularized adhesions to the body wall did not become tumorous and such animals did not become anestrous. Because the uteri and vaginas of mice with large tumors are not atrophic it is assumed that the liver is unable to destroy the hormones that the tumors produce or that the hormone produced is qualitatively of such a type as to be unchanged by the liver.

How can one account for these observations? It is thought that they afford an example of the tumorigenic action of specific pituitary growth-stimulating hormone, namely, the follicle-stimulating hormone. As mentioned previously the hormone content of the pituitary gland and the excreted gonadotrophic hormones increase subsequent to castration, and the increase involves primarily the follicle-stimulating hormone. Ovaries transplanted into the spleen continually have their endocrine secretions inactivated by the liver before they reach the general circulation and, hence, before they reach the pituitary gland. The vaginal smears of such animals rarely show cornified cells characteristic of estrus. Under such circumstances the mouse has an ovary but at the same time it is physiologically castrated. The continuous stimulation of the ovary by an augmented and continuous follicle-stimulating-hormone leads to abnormal proliferation and neoplasia. Injections of estrogenic and androgenic hormones in mice bearing intrasplenic transplants both prevent ovarian tumorigenesis (24).

This is another excellent example of a tumor arising because of the creation of an abnormal endocrine environment. Furthermore it is possible that the same mechanism is effective in the genesis of ovarian tumors subsequent to x-irradiation. Under such circumstances the hormone-producing capacity of the ovary may be so impaired that the pituitary secretion cannot be kept under control. More experiments must be done before the various assumptions can be accepted as facts.

SUMMARY

Tumors of four endocrine glands arise under conditions of hormonal imbalances in experimental animals. The gonadotrophic hormones of the pituitary, in part by experimentation and in part by assumption, have been associated with tumors of the testes and ovaries. Pituitary chromophobe adenomas develop in estrogen-treated mice of some strains but not in others and the tendency to acquire such tumors is transmitted by both male and female mice to their hybrid offspring. An environment deficient in gonadal hormones and high in gonadotrophic hormone (F.S.H.) results in adrenal cortical tumors in mice of some strains and in hyperplastic growth of the adrenal cortex in mice of other strains.

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The Influence of Sex Hormones on Mammary Tumors Induced by 2-Acetaminofluorene*

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There is abundant evidence that estrogens enhance the development of mammary tumors in high-cancer strains of mice, with the possible exception of the Marsh-Buffalo strain. The inhibitory effect of testosterone in this connection is also well known. Although similar studies in species other than the mouse have yielded conflicting results, there is evidence that estrogens enhance the development of mammary tumors in rats.

The present report deals with the influence of estrogen, androgen and progesterone upon the development of mammary tumors in rats receiving 2-acetaminofluorene in their diet.

MATERIAL AND METHODS

Observations were made on 179 virgin female and 196 male rats about 2 months old; the majority were of the Sherman strain, the remainder Wistar descendants. We have encountered no significant difference between these two groups in their response to acetaminofluorene or to the various sex hormones employed in this and previous studies (8, 21). All received 0.03 per cent 2-acetaminofluorene in the following diet (protein content 16 per cent) to the time of sacrifice: corn meal, casein, alfalfa, linseed oil, bone ash, and NaCl, with supplements of brewers' yeast and cod liver oil. They were divided into the following experimental groups:

Control Group.—57 females and 79 males received no additional treatment.

Endogenous Estrogen Group.—17 females received 25 I. U. of pregnant mare serum (PMS) gonadotrophin (in aqueous solution) intramuscularly 3 times weekly.

Exogenous Estrogen Group.—36 females and 52 males received 0.125 mgm. of estradiol dipropionate (in sesame oil) intramuscularly 3 times weekly.

Exogenous Androgen Group.—32 females and 51 males received 0.5 mgm. of testosterone propionate (in sesame oil) intramuscularly 3 times weekly.

Progesterone Group.—26 females, 11 castrated females and 14 males received 0.5 mgm. of progesterone intramuscularly 3 times weekly.

Treatment was continued in all instances to the time of death or sacrifice. The control animals were examined weekly and the hormone-treated animals at the time of injection for the presence of mammary tumor. Vaginal smears were made repeatedly during the experimental period and in every instance at the time of sacrifice. The mammary tumors were fixed in Bouin's solution and sections were stained with hematoxylin-eosin and by Masson's trichrome method.

RESULTS

The incidence of malignant tumors of the breast in the several groups is indicated in Table I. The time of appearance of the first palpable tumor in each group, subsequently confirmed histologically, was as follows: female control, 159 days; female-estradiol, 161 days; female-gonadotrophin, 285 days; female-progesterone, 93 days; male-estradiol, 177 days; male-testosterone, 141 days. It is interesting that, in contrast to liver tumors (8, 21), there was apparently no tendency for the incidence of breast tumors to increase with increasing duration of treatment in any of the experimental groups.

The tumors were either single or multiple, unilateral or bilateral. When small, they were usually firm; when large, they were often cystic, containing either hemorrhagic or creamy, gray-yellow fluid. The smaller tumors tended to be encapsulated and could be shelled out readily. Larger tumors were usually attached to the skin, which was often ulcerated. The surface was irregularly lobulated, pale gray, frequently with bluish cysts. The cut surface was irregularly lobulated, mottled yellow-gray in color, with cystic spaces containing bloody or creamy fluid.

There were certain differences in the histologic

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TABLE I: INCIDENCE OF MAMMARY CARCINOMA

FEMALE

Days of treatment	Control No. of rats	Control Tumor	Estradiol No. of rats	Estradiol Tumor	PMS Gonadotrophin No. of rats	PMS Gonadotrophin Tumor	Testosterone No. of rats	Testosterone Tumor	Progesterone No. of rats	Progesterone Tumor	Castrate Progesterone No. of rats	Castrate Progesterone Tumor
92–200	25	9	18	3*	2	0	21	0	21	21	11	0
201–250	8	2	9	5	3	0	7	0	2	0		
251–300	3	1	8	0	10	1	4	0	1	1		
301–350	11	4	1	0	2	2			2	0		
351–400	10	1										
Total	57	17 (30%)	36	8 (22%)	17	3 (18%)	32	0	26	22 (85%)	11	0

MALE

Days of treatment	Control No. of rats	Control Tumor	Estradiol No. of rats	Estradiol Tumor	PMS Gonadotrophin No. of rats	PMS Gonadotrophin Tumor	Testosterone No. of rats	Testosterone Tumor	Progesterone No. of rats	Progesterone Tumor	Castrate Progesterone No. of rats	Castrate Progesterone Tumor
92–200	34	0	36	3			17	1	6	0		
201–250	23	0	16	0			18	0	8	0		
251–300	14	0					10	0				
301–350	8	0					6	0				
351–400												
Total	79	0 (0%)	52	3 (6%)			51	1 (2%)	14	0		

* One sarcoma

characteristics of the tumors in the various experimental groups.

Control Group.—The malignant proliferation involved both epithelial and connective tissue cells, their relative proportions varying in different portions of the tumor, with epithelial proliferation generally dominant. The pattern most commonly observed was that of adenocarcinoma (Fig. 1). This was characterized by acini of varying size, lined by cuboidal or low columnar epithelial cells with dark nuclei and vacuolated or granular cytoplasm, occasionally containing pink homogeneous material in their lumen. Groups of these acini were separated by connective tissue septa containing lymphocytes and fibrocytes.

The type of lesion next in order of frequency in the control group was papillary cystadenoma. This was characterized by irregular cystic spaces, containing blood or albuminous fluid, lined by low, dark, cuboidal cells, with numerous papillary infoldings comprising a connective tissue core covered by layers of tall columnar epithelium (Fig. 3). Another lesion seen frequently in this group was characterized by papillary projections into dilated ducts, resembling intraductal papilloma (Fig. 2). Ductal carcinoma (comedo) (Fig. 4) occurred less frequently than adenocarcinoma and papillary cystadenoma.

Areas of necrosis and hemorrhage were frequently seen in the larger nodules, as well as greatly distended blood vessels filled with red blood cells. Lesions of all types contained numerous mitotic figures and their malignant nature was evidenced by invasion of adjacent muscle (Fig. 6), infiltration of the capsule and metastasis to the liver and lungs. The amount of connective tissue varied, but at times the proliferation of connective tissue was so extensive as to resemble the scirrhous form of mammary carcinoma (Fig. 5). In such cases the tissue was usually quite cellular, containing numerous lymphocytes and fibrocytes, with scattered, compressed groups of dark-staining epithelial cells in acinous arrangement.

Hormone-Treated Groups.—The mammary tumors in females receiving estradiol, gonadotrophin or progesterone were almost exclusively of the adenocarcinoma and papillary cystadenoma varieties, both of which occurred practically always in the same tumor. A few in the progesterone group (but none in the estrogen series) contained also lesions characteristic of ductal carcinoma. There was no essential difference between the histologic characteristics of the lesions in the estrogen-treated and progesterone-treated females. Both differed from the control group, however, in that connective tissue proliferation was relatively slight except in one animal receiving progesterone (Fig. 5). One mammary sarcoma occurred in the estrogen group. The single mammary tumor in the testosterone-treated males differed in no respect from those seen in the estrogen and progesterone female groups, except that it, and also the tumors in the estrogen-treated males, exhibited only acinar epithelial proliferation, with no evidence of duct involvement.

The acinar cells in the hormone-treated groups were cuboidal or columnar, their cytoplasm often

being vacuolated or foamy. The lumen of the acini and cysts consistently contained pale-blue amorphous or pink granular and vacuolated material and desquamated cells. The stroma of the tumors in the progesterone-treated animals contained areas which appeared edematous or myxomatous. Not only was the tumor incidence much higher in this group (85 per cent), but also the growth of the tumors was much more rapid than in the other groups.

DISCUSSION

There is considerable variation in the reported incidence of mammary carcinoma in female rats of various strains receiving 2-acetaminofluorene, as follows: Bielschowsky (2, 3), Wistar 64 per cent, piebald 4 per cent; Harris (11), Wistar 62 per cent; Wilson, De Eds, and Cox (24), Slonaker 8 per cent; Dunning, Curtis, and Madsen (9), Marshal none, Copenhagen none, August 10 per cent, Fischer 20 per cent, A × C 10 per cent. The difference may be attributable in some instances to differences in diet and in dosage of the carcinogen, but this is not the case with the two strains employed by Bielschowsky (2, 3) nor the four strains studied by Dunning, Curtis, and Madsen (9). However, the fact that there were only 10 animals in each of the groups reported by the latter raises some question as to the statistical significance of the observed variation (0 to 20 per cent). Bielschowsky (3) reported the occurrence of mammary cancer in 1 of 11 female castrates (9 per cent) and in 3 of 41 males (4 per cent). No breast tumors in acetaminofluorene-treated male rats have been reported by other investigators and none has been observed by us in male rats receiving the carcinogen alone. In our group of female controls (which received only acetaminofluorene) the incidence was 30 per cent, with no significant difference between Sherman and Wistar animals.

The well-established formula for development of spontaneous mammary cancer in inbred strains of mice includes three factors: (a) genetic susceptibility, (b) the milk agent and (c) hormonal stimulation of mammary growth. Studies of the induction of breast tumors in mice by methylcholanthrene and 1,2,5,6-dibenzanthracene indicate that the first two of these factors are not essential under these circumstances (14, 15, 20, 22). Although certain strains are more resistant than others to the induction of mammary cancer by methylcholanthrene, this variation could not be correlated with susceptibility to development of spontaneous mammary cancer (14). The hormonal factors are, however, operative. It would appear that the breast must attain a certain degree and type of growth before either the milk agent or the carcinogens mentioned above can evoke the neoplastic reaction (20).

All stocks of rats studied have a very low incidence of spontaneous mammary cancer. Administration of even large amounts of estrogen usually results in the development of only benign lesions. Nevertheless, there are a few reports of the induction of mammary cancer in rats exposed to estrogen for prolonged periods, *e.g.*, by means of pellet implantation (10, 17). It has been suggested that the apparently high resistance of rats in this connection may be due to lack of a proper genetic background or to the possibility that the estrogen requirement for induction of mammary cancer is much higher for rats than for mice.

The sex difference in incidence of breast tumors following administration of 2-acetaminofluorene is in accord with these observations and with prevalent views regarding the necessity for hormonal, *i.e.* presumably estrogenic, stimulation of mammary growth as a prerequisite for mammary carcinogenesis. The inhibitory influence of testosterone and castration in females was also to have been anticipated on this basis. However, in the case of spontaneous tumors in high-tumor strains of mice, with few exceptions, the incidence is low in nonbreeding as compared to breeding females, increasing to that in the latter after administration of estrogen (20). Similarly, in the case of methylcholanthrene-induced breast tumors in mice, the incidence has been reported to be low in nonbreeding as compared to breeding females (16), although Orr (19) reported a high incidence in non-breeding females of low-tumor strains (IFS and CBA). The findings in acetaminofluorene-treated rats are not in agreement with the former (16), inasmuch as the females were virgins and this carcinogen is not estrogenic (2). The high incidence of mammary tumors in non-breeding females, viewed in the light of their absence in males and in castrate and testosterone-treated females, suggests that whereas estrogen is essential, only relatively small amounts are required for the development of such tumors under the influence of 2-acetaminofluorene. If this be the case, one cannot invoke the hypothesis of a high requirement, in this connection, of estrogen to account for the failure of estradiol (22 per cent) and gonadotrophin (18 per cent) to increase the tumor incidence in females and the relatively slight effect of estrogen in males

(6 per cent). The occurrence of a mammary tumor in one male rat receiving testosterone (141 days) is difficult to explain in view of the inhibitory influence of this hormone in females.

The observation that progesterone exerts such a remarkable effect in increasing the incidence (85 per cent) and rate of growth of mammary cancer in females raises the question as to whether this substance, rather than estrogen, may be the limiting hormonal factor in the genesis of this tumor under the existing experimental conditions. No such effect of progesterone has been reported previously, with the exception of the observation of a relatively high incidence of mammary tumor in progesterone-treated pregnant mice of a low-tumor strain (23). It seems noteworthy, also, that in this group, as in the other experimental groups, the incidence of tumor did not increase with prolongation of the period of treatment. Other studies of the influence of this agent upon spontaneous mammary cancer in mice (5, 7, 13) and mammary fibroadenoma in rats (12, 18) have revealed either no effect or, more frequently, inhibition of growth of the tumors. We are aware of no previous reports of its use in conjunction with chemical carcinogens capable of inducing mammary tumors.

No satisfactory explanation of this phenomenon is readily apparent. There are several observations that the combined action of estrogen and progesterone is necessary for maximum proliferation of the alveolar system in the mouse and rat. However, there is no clear evidence that administration of progesterone alone produces a significant stimulating effect upon the mammary gland of normal female rats. The absence of breast tumors in progesterone-treated males and castrate females indicates that some estrogen is required for the production of the stimulating effect of progesterone upon mammary carcinogenesis under the influence of acetaminofluorene. As noted previously, the one instance of tumor in the testosterone-treated males is difficult to explain, although there is evidence that testosterone stimulates lobule growth in immature male and castrate female rats (1). The remarkable tumor-stimulating effect of progesterone in females, viewed in conjunction with the lack of such effect of estrogen in females and its minimal effect in males (6 per cent tumors), suggests, as indicated previously, that in the presence of a necessary, but relatively small amount of estrogen, the quantity of progesterone is the factor that determines the development of mammary cancer by 2-acetaminofluorene. Studies are in progress of the effect of administration of estrogen and progesterone simultaneously to males and castrate females receiving 2-acetaminofluorene. Data obtained in these experimental groups should indicate whether or not this hypothesis is valid.

Although the nature of our material does not permit any conclusion as to the histogenesis of the tumors observed, certain points of similarity to and difference from other types of experimental and spontaneous breast tumors seem worthy of mention. The histologic characteristics of the tumors in our control (AAF) group conformed to those described by Bielschowsky (4), who stated that (a) nearly all were adenocarcinoma, (b) malignant intraductal papilloma occurred more commonly than in spontaneous mammary tumors in mice, (c) metastases occurred only when tumors had reached considerable size, and (d) squamous metaplasia was seen rarely (not at all in our series) and scirrhous tumors infrequently. Papillary cystadenoma apparently occurred more commonly in our series.

It has been pointed out in studies of mammary tumors in mice that these differ from human breast cancer in the infrequency of true duct carcinoma and intraductal papilloma (6). On the other hand, duct carcinoma was observed frequently by Nelson (17) and Astwood, Geschickter, and Rausch (1) in estrogen-induced mammary tumors in rats. In view of these observations, the high incidence of these ductal lesions in rats receiving 2-acetaminofluorene alone and its absence in those receiving estrogen simultaneously suggest that there is some fundamental difference not only in the mode of action of these two agents but also in the effect of each in the presence of the other. The lesions induced by acetaminofluorene differed also from those induced in mice by methylcholanthrene (15) in that the squamous metaplasia commonly observed in the latter was not encountered in any experimental group in our series.

SUMMARY

The incidence of mammary carcinoma in nonbreeding female Sherman and Wistar rats receiving 2-acetaminofluorene was not increased by simultaneous administration of estrogen but was increased enormously by progesterone. Breast tumors, absent in carcinogen-treated males, appeared in a small percentage receiving estrogen and in one receiving testosterone.

The data suggest that small amounts of estrogen are probably necessary for the development of breast carcinoma in Sherman and Wistar rats treated with 2-acetaminofluorene, but that the

quantity of progesterone may be the limiting hormonal factor in this connection.

The morphologic characteristics of these lesions are compared with those of spontaneous and carcinogen-induced tumors in mice and estrogen-induced tumors in rats.

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DESCRIPTION OF FIGURES 1 TO 6

Fig. 1.—Adenocarcinomatous portion of the mammary tumor of male rat treated with testosterone for 141 days. Note the presence of pale vacuolated cells and pink granular material in the lumen of the dilated acini. Mag. × 100.

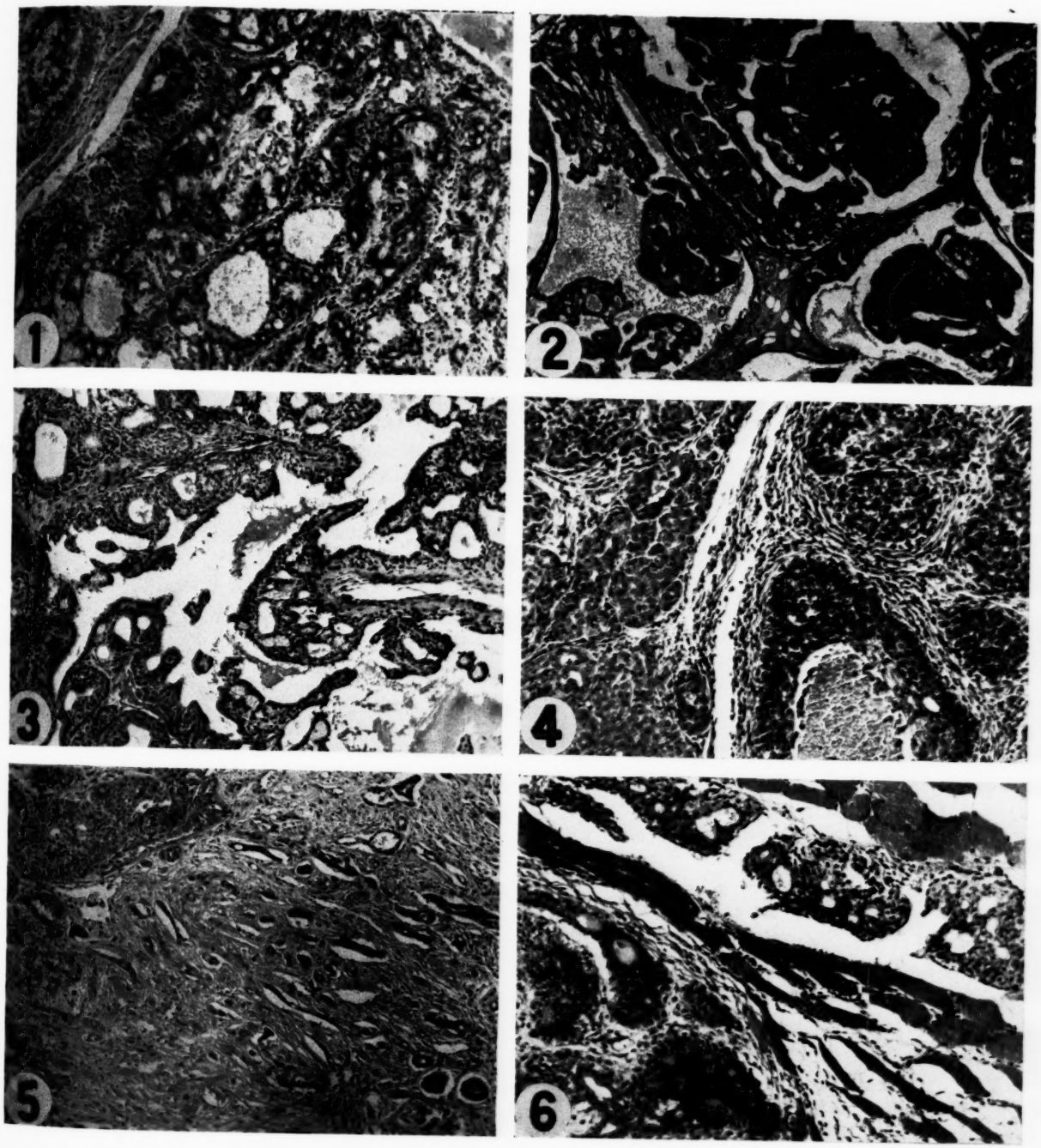
Fig. 2.—Portion of mammary tumor of a control female rat (carcinogen alone) treated for 159 days. Note the numerous ductal papillomata. Mag. × 50.

Fig. 3.—Papillary-cystadenocarcinomatous portion of a mammary tumor of a female rat treated with progesterone for 108 days. Mag. × 70.

Fig. 4.—Portion of duct or "comedo" carcinoma from a control female rat (carcinogen alone) treated for 340 days. Mag. × 125.

Fig. 5.—Portion of the mammary tumor of female rat treated with progesterone for 291 days. Note the marked proliferation of the connective tissue compressing the adenomatous structures. This type of lesion was more common in the control animals and resembles scirrhous adenocarcinoma. Mag. × 60.

Fig. 6.—Another portion of the tumor of Fig. 3, revealing the infiltrative growth between the adjacent skeletal muscle fibers. Mag. × 100.



FIGS. 1-6

Early Changes in the Experimentally Produced Adenomas and Adenocarcinomas of the Stomach*

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Until the work of Stewart and Lorenz in 1942 (7), attempts at producing adenocarcinomas of the stomach by means of the carcinogens had failed. Klein and Palmer reviewed these efforts in 1941 (3). Carcinogens had been administered with the diet mixed with roughage, placed in the mouth by dropper and kept within the lumen of the stomach for some time by means of paraffin pellets. Squamous-cell carcinomas were produced in the lower end of the esophagus and in the forestomach of rats and mice, but not adenocarcinomas of the glandular portion. The forestomach is lined with squamous cell epithelium and theoretically is not protected by mucin as is the rest of the stomach.

Stewart (6) placed the carcinogen within the wall of the stomach of mice by means of threads, oil, and horse serum. The greatest production of adenocarcinomas was obtained with a suspension of methylcholanthrene in horse serum. Of 47 mice injected with this suspension, 6 developed adenocarcinomas; 12, mixed adenocarcinomas and sarcomas; 6, adenocanthomas; 2, mixed adenocanthomas and sarcomas; 7, adenomas; and 3, sarcomas. The lesions were produced within 19 to 46 weeks.

In this investigation, threads impregnated with methylcholanthrene were used to produce adenocarcinomas and adenomas, and the early changes occurring about them were studied in an attempt to disclose the mechanism of formation of new growths in the stomach. The reactions of the cells of the stomach wall to unimpregnated threads and to threads containing a similar but noncarcinogenic anthracene compound were also studied in order to distinguish unusual changes about the threads containing the carcinogen. Previously, a comparison was made of early tissue reactions about carcinogenic and noncarcinogenic threads in muscle and skin in the formation of sarcoma and epithelioma (2).

METHOD

Untreated Corticelli silk thread, size B, was placed over methylcholanthrene and the powder was melted by heating. The thread was stirred into the liquid, which was subsequently allowed to recrystallize by cooling. Melting and stirring were repeated until the thread became swollen and yellow with the carcinogen. The noncarcinogenic compound was put into the thread by the same method. 1,2-Benzanthracene was used as the similar anthracene but noncarcinogenic compound. Nine investigators have failed to produce malignant tumors with this compound (1).

Rats of the Wistar strain were employed. Under ether anesthesia, an incision was made through the midline of the anterior abdominal wall and the stomach was delivered into the wound. By exerting tension on the pylorus, a fine straight needle could be pushed under the serosa towards the esophagus for a distance sufficiently great to allow the insertion of 1 cm. of thread. Its ends were cut just as they pierced the serosa; no long ends were left because they produce sarcomas outside the stomach. Threads placed in this manner lay between the serosa and mucosa, under the central or acid glandular portion of the stomach. The abdominal wall was closed with silk sutures.

The rats were fed on equal parts of Rockland rat pellets and Purina dog chow. Three rats were killed at weekly intervals for 35 days in order to study the early changes, then at 14 day intervals for 120 days. Others were killed from the 200th to 590th days when tumors could be palpated. A companion rat exposed to the carcinogen for the same length of time but without a palpable tumor was killed each time to ascertain why a tumor had not developed. In all, approximately 150 rats were used.

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RESULTS

Figs. 1 and 2 illustrate adenocarcinomas and adenomas produced by the carcinogenic threads.

The early changes preceding the formation of these tumors were as follows: Within a week after implantation, a ridge of heaped-up epithelium overlay the course of the threads. These ridges tended to ulcerate as a result of an inflammatory change. Within 72 hours after insertion of a carcinogenic thread, a heavy infiltration of round cells took place about it; blood vessels became dilated and more numerous at the periphery of the area of infiltration, edema occurred, and finally ulceration resulted (Fig. 3). Within 2 or 3 weeks, epithelial-lined clefts or sinuses formed in these ridges. Sinuses formed in the following manner: a small amount of thread became exposed as the result of ulceration in a ridge, or one end of a thread sloughed out into the lumen, or a thread perforated the mucosa during insertion. In either case, epithelial cells grew back along the portion of the thread that remained embedded, down along its course through the muscularis mucosæ, and filling the tract. Often both perforation and ulceration worked together to produce sinuses.

Some ulcerations developed as early as 10 days after a thread was embedded, and eventually exposed all carcinogenic threads. For example, after 150 days, silk fibers were rarely found in any of the microscopic sections, indicating that either the threads had sloughed out or they had been easily removed by the microtome during cutting and therefore were not well encapsulated in fibrous tissue.

Clefts formed whenever the mucosa was destroyed over a long length of thread and yet it remained long enough within the stomach wall to allow the defect to become relined with new epithelium. Grossly, clefts were paralleled on either side by ridges of hypertrophied mucosa, and when these ridges were pulled apart in some of the early specimens, the thread could be seen within the depth of the cleft. When a cleft did not extend for the entire length of a thread, sinuses formed on either end of it.

The new epithelium that grew into and lined these sinuses and clefts came from the surrounding mucosa. As soon as a break occurred in the muscularis mucosæ, the superficial mucus-secreting cells extended downward in a single row. They quickly formed acini in the sinuses and completely filled the tube-like defect with a cylinder of acini by the 26th day, as shown in Fig. 4. Around the circumference of the cylinder a very thin rim of reticulin was deposited, but no reticulin was deposited between the acini. These mucous cells, extending in a single row, first relined the inner circumferences of the clefts and then proliferated to form a new mucosa, less thick than the original and with rudimentary crypts (Fig. 5). Beyond it a fairly deep layer of reticulin was deposited. The morphology of this new epithelium that now existed below the muscularis mucosæ resembled more that of the prepyloric antrum of the stomach than that of the acid pepsin-secreting epithelium of its origin. Special stains for pepsin failed to color this epithelium, indicating that this enzyme was not secreted. No eosin-staining acid cells were seen.

Instead, in both the sinuses and clefts, the epithelial structures were composed of cuboidal cells that took a basophilic stain. The cells were not irregular in size nor contour, nor did they contain an unusual number of mitotic figures. Many of them stained with mucicarmine and some contained pink droplets, indicating the presence of mucus. Occasionally they formed mucous cysts. No further change occurred in the morphology of these misplaced epithelial structures until after the 35th day.

Ulcerations sometimes occurred about the control threads impregnated with 1,2-benzanthracene but sloughing did not take place as rapidly, as frequently, or as completely. In fact, about half of these threads finally became encapsulated. When ulceration occurred, however, or when one of these threads perforated the mucosa, the superficial mucosal cells likewise grew downward in the manner just described to form epithelial-lined clefts and sinuses. In the sinuses acini formed and inside the clefts mucus-secreting cells later proliferated to form a new epithelium. Both architectures persisted thereafter without subsequent change. Neither pepsin nor acid-secreting cells ever reappeared among these cells that were observed to continue to exist below the muscularis mucosæ for as long as 150 days. In some instances they did become slightly atrophic.

The downgrowths of epithelial cells around the 1,2-benzanthracene threads also became encapsulated with reticulin. On cross section a mass of acini could be seen surrounded with a capsule of reticulin and in one quadrant of the circle, fibers of silk were found separately encapsulated with reticulin. Giant cells were present between them. This arrangement represented the final quiescent stage of reorganization. Even when a downgrowth of epithelium was present, encapsulation of the thread containing 1,2-benzanthracene was complete in one month.

Clefts did not form about untreated control silk threads although sinuses occasionally formed when a length of thread perforated through the mucosa or an end extended into the lumen. Then epithelium grew back along the untreated silk in exactly the same manner as it did along the carcinogenic and non-carcinogen threads. Sinuses containing acini formed and both the thread and the epithelial tube finally became encapsulated. The process was completed about 10 days earlier than about the 1,2-benzanthracene threads. Usually the untreated silk threads became completely encapsulated without a downgrowth of surrounding epithelium. The timing of the process and the arrangement of reticulum about each fiber has been described in a previous report (2).

In other words the mucus-secreting cells extended downward early around all threads under the stimulus of injury or when a break occurred in the muscularis mucosæ. However, they continued to proliferate and to form adenomas or adenocarcinomas only in the presence of methylcholanthrene. The invasive tumors formed in the sinuses while adenomas formed in the clefts.

The transformation in the sinuses will be described first. Until the 35th day the tubes of epithelium extending downward about the methylcholanthrene threads were somewhat larger than those about the controls, possibly as the result of a larger amount of tissue destroyed by the carcinogen. Save for this difference, the epithelium could not be distinguished from that seen about the chemical control. By the 50th day, on the other hand, differences were becoming apparent. Whereas each fiber of the thread with 1,2-benzanthracene was encapsulated separately and 8 to 10 acini of epithelium appeared in a perfect circle on cross section, with regular and heavy strands of reticulin deposited around the periphery of the group; by contrast, the thread containing methylcholanthrene was not encapsulated at all. Many more acini were present and there were many mononuclear cells between the acini and about their periphery but not between the fibers of the thread. The acini were not arranged in a perfect circle. They were distributed irregularly and some jutted out into an imperfect reticulin barrier of very fine fibrils about the periphery. In these areas where the epithelial cells extended, the fibrils were edematous and infiltrated with mononuclear cells.

Grossly, there was considerable thickening of the stomach wall for some distance about the thread. (Fig. 6). The increase in the number of acini and their extension outward suggested that by the 50th day the epithelial cells were beginning to proliferate again and that by some mechanism, recently established reticulin barriers were destroyed and new ones were not being deposited.

Thereafter with the passage of time, the masses of acini enlarged. They became 10 or 12 times their original size at 35 days, they extended through zones of weakened reticulin clear out to the liver and in areas, the cells grew in compact groups. Some of them also became irregular in size and shape and mitotic figures became more numerous. In two animals large tumors grew outside the stomach and there were peritoneal implantations locally, but no metastases were found in the liver or lungs.

The adenomas formed in the clefts as the result of further proliferation of the epithelium that lined them. Papillomatous growths began to appear after the 50th day and later; they not only filled the cleft but pushed open its mouth and grew by long stalks into the lumen of the stomach. Normal mucosa on either side was pushed far apart by growths bulging through the mouth of a cleft (Fig. 7). The reticulin barrier, previously deposited outside the lining epithelium of the cleft, was undamaged by the new growth. In other words the growth of the adenoma was outward into the lumen of the stomach and not into the tissues. In some of the largest adenomas, however, the component cells were irregular in size and shape and this change, combined with a wide base, suggested the appearance of a fungating carcinoma.

In summary, then, 3 distinct changes were observed in these experiments in the formation of the new growths of the stomach. First, static tissues: muscle, reticulin, fat and certain types of epithelial cells—the chief and parietal cells were destroyed by the carcinogen; second, mucus-secreting cells survived this destruction and extended from the surface of the mucosa down through the normal connective tissue barrier into the space left by the destruction, and continued to exist without organized blood supply. Lastly, under the continuing influence of the carcinogen, these cells proliferated further to form large masses of epithelial tissue. When adenocarcinomas developed, newly formed reticulin barriers were destroyed and attempts to form new barriers failed. On the other hand, if the reticulin barrier remained intact, an adenoma formed.

Of course, not all epithelial-lined clefts or sinuses formed by methylcholanthrene threads developed adenomas or adenocarcinomas. In the first group of animals, for example, only 6 out of 22

that were allowed to run long enough developed new growths. In these, size B silk was impregnated with the carcinogen. In a second group, a larger silk thread, size C, was used and no new growths developed because all threads sloughed from the stomach wall within 3 weeks. In a third group, B silk was again used, but the ends were not impregnated with chemical so that the untreated portions could become encapsulated and thus keep the entire thread in contact with the ingrown epithelium for a longer period of time. In this lot 14 out of 40 had invasive epithelial growths and 9 had adenomatous growths, illustrating the importance of persistence of contact of the carcinogen with the misplaced or buried mucous cells. Seven sarcomas were found in the three series and only 2 sarcomas were combined with epithelial growths. In the stomachs of 2 animals large growths of acini were found within the stomach wall and each acinus was separately surrounded by heavy fibers of collagen. So many acini were present that the thread must have remained for a sufficient length of time to cause further proliferation of the acini, yet the effect of the methylcholanthrene was eventually lost and the entire area became filled with dense connective tissue fibers, as may be seen in Fig. 8.

DISCUSSION

Certain tissues and cells composing the stomach wall were destroyed by the carcinogen. Muscle, leukocytes, parietal and chief cells, reticulin and collagen were destroyed. So extensive was the damage that the carcinogenic threads sloughed from the tissues. On the other hand, mononuclear cells and fibroblasts survived at a distance from the thread and, more important still, the superficial mucus-secreting cells not only survived, but they moved downward into the area containing the carcinogen and proliferated there.

Similar extensions of mucosal cells also were found growing downward below the muscularis mucosæ; occasionally around untreated threads and more frequently around threads containing a similar but noncarcinogenic anthracene compound. Because these downgrowths occurred with injury alone—but always with one producing a defect in the muscularis mucosæ—we conclude that even when the carcinogen was present the process was initiated by non-specific injury and that removal of the barrier of the muscularis mucosæ was the important factor allowing the mucus-secreting cells to move downward and to organize themselves in a misplaced growth.

The initial movement of these cells in a single row recalls the outward movement in a single row of epithelial cells occurring in the basement layer of the skin after injury. In the wound of the skin this movement is attributed to amoeboid motion and is combined with hyperplasia of the old uninjured epithelium on either side of the defect. Hyperplasia and hypertrophy of the surrounding superficial mucosal cells also occurred in the stomach, accounting for the two parallel ridges of mucosa overhanging either side of a cleft. Because of these similarities, the initial movement of mucous cells must likewise be attributed to amoeboid motion.

When no carcinogen was present, these mucous cells continued to exist below the muscularis mucosæ and showed no tendency to proliferate further. Instead, they achieved some degree of differentiation and became encapsulated. The sinuses formed anatomically resembled those described originally by Aschoff-Rokitansky in the stomach, duodenum and gallbladder of man. The persistence of mucous cells under these circumstances illustrates their capacity to survive, for then they normally are farthest removed from blood channels and therefore receive their nutrition tenuously, while at the same time acting as buffer cells against injury. Conversely, the relative incapacity of the acid and pepsin-secreting cells to survive injury and to proliferate thereafter is demonstrated.

Displacement of mucous cells beneath the muscularis mucosæ in the presence of a carcinogen seems essential for the formation of a new growth in the stomach. In this new environment mucous cells could alter their function of secreting highly protective mucus, surrounding reticulin barriers could be further destroyed and persistent contact with the carcinogen becomes possible.

Neither these three alterations nor the change in blood supply could have been present in earlier experimental attempts to produce gastric neoplasms with the carcinogens. Their absence may explain why feeding and placing carcinogens in contact with normally secreting mucous cells have repeatedly failed to produce gastric tumors. The mucus-secreting cells lining the innermost surface of the stomach are ordinarily continuously subjected to injury and they, in response, desquamate and regenerate. In the presence of the carcinogen within the lumen and increased surface destruction, these normal activities would only be intensified. Destruction of reticulin barriers seems to be particularly important because this destruction opens pathways for cells to move, *i.e.*, to become invasive

and autonomous in growth. Lastly, the thesis that destruction within the stomach wall is necessary to produce new growths has historical confirmation—the first successful attempt to produce carcinoma of the stomach was obtained by Stewart and Lorenz (6) when the carcinogen was placed within the wall, in contrast with the many previous failures resulting from the maintenance of carcinogen in the lumen. Surface application of a carcinogen, in fact, produces neoplasms in only one tissue, the epidermis, yet in this structure the carcinogen dissolves in the sebum and penetrates into the skin so that changes are instituted deep within the tissue (5). When the carcinogen fails to penetrate, neoplasms do not form (4). Acceptance of the thesis that destruction within the wall is necessary focusses attention on the important corollary that the carcinogen must institute changes other than causing cells to proliferate.

The experiments furnished some clue to the duration of contact required between the tissues and the carcinogen. If the carcinogenic thread remained within the stomach wall for a period less than 35 days, no attempt at tumor formation was observed. Stomachs of rats have been reopened and the threads removed if they had not sloughed away by the 35th day and no growths have resulted. Threads removed after the 60th day, on the other hand, yielded adenomas.

Lastly, the mucous cell of the gastrointestinal tract must be added to the list of those cells that can survive the destructive action of carcinogens. Like the fibroblast and the epithelial cell of the basement layer of the skin, it is capable of ameboid motion and can, therefore, move away from a focus of destruction. Like them also it possesses unusual proliferative capacities normally, and is constantly regenerating in response to destructive forces. By contrast, and equally as important, is the fact that the static elements of the tissues, particularly reticulin and collagen, cannot move away from destruction and can be elaborated only secondarily after cells have matured and would not regenerate despite continuing specific destruction.

Naturally, after the completion of these experiments, we were curious whether the evolutionary changes that had been observed in the experimental production of new growths of the stomach could also be traced in neoplasms evolving spontaneously in the human stomach.

Loss of the capacity to secrete acid and pepsin, or mucous transformation, atrophy or "intestinalization" is a common precancerous change. Its presence in the mucosa of stomachs resected for cancer and for peptic ulcer has been statistically correlated by Stout (8). Through his courtesy, approximately 250 microscopic sections from resected stomachs were reviewed to discover if there were more changes at a distance in the muscularis mucosæ in carcinomatous stomachs than in those resected for ulcer.

The following observations were made: (a) The muscularis mucosæ is nearly always completely destroyed at the site of the carcinoma. (b) Lymphoid infiltration into the muscularis mucosæ is not uncommon, but the epithelial cells do not transgress through these areas. (c) Aschoff-Rokitansky sinuses are sometimes found but are not frequent, and the epithelial cells that inhabit these sinuses are mucous cells only. (d) In superficially spreading carcinoma, small cells that tend to grow in sheets above the muscularis mucosæ seem to drop right through the muscularis mucosæ and into the loose areolar connective tissue beneath, but no obvious break in the muscularis mucosæ can be detected.

CONCLUSIONS

Silk threads containing methylcholanthrene produced new growths when embedded in the wall of the rat's stomach. The early changes have been studied. Shortly after implantation beneath the serosa, round cells infiltrated and edema appeared. Ulceration occurred in the mucosa and then the superficial mucous cell on either side grew into the defect to form an epithelial-lined cleft. If a thread perforated the mucosa or the ulceration occurred directly over a thread that remained in position, the superficial mucous cells grew back along the thread to form an epithelial-lined sinus. Acid and pepsin cells were destroyed and did not grow downward. In the clefts these cells formed a new mucosa composed entirely of mucous cells while the sinus was filled with a cylinder of these cells that arranged themselves in acini. This process took approximately 2 weeks and the cells showed no malignant change.

After 35 days, the cells that were able to continue their existence beneath the muscularis mucosæ began to grow again. Adenomas formed in the clefts while adenocarcinomas formed in the sinuses. Adenomas grew outward toward the lumen of the stomach, pushed open the mouth of the cleft, and sometimes filled the lumen of the stomach. This type of growth did not disturb the reticulin boundary that had been formed beyond the new mucosa lining the cleft. Adenocarcinomas grew within the wall of the stomach and the re-

ticulin barriers already formed about the sinus were destroyed.

These mucus cells transposed beneath the muscularis mucosæ required prolonged contact with the carcinogen or else they remained in the state typical of their orientation in the sinus or cleft. This contact in the rat must exist at least for 35 days.

The reasons why placing the carcinogen in the wall of the stomach is successful in producing new growths in contrast to failures obtained by keeping it in the lumen is discussed. In the wall of the stomach long contact can be maintained with mucous cells, existing in a new environment where perhaps their protective secretion may be lost and where damage can be done by the carcinogen to the surrounding reticulin barriers and blood vessels. Carcinogens in the lumen by contrast only make contact with normal mucus cells that desquamate and increase in their rate of proliferation.

The mucus cells must be added to the list of cells able to survive destruction caused by the carcinogens. Like the fibroblast and the epithelial cell of the basement membrane of the skin, it possesses amoeboid motion and an inherent high rate of proliferation in response to injury.

Microscopic sections of the human stomachs resected for carcinoma and ulcer were examined for the presence of mucous transformation, Aschoff sinuses and destruction of the muscularis mucosæ to ascertain whether a correlation could be obtained with the experimental results. Mucous transformation and destruction of the muscularis mucosæ directly over the tumor were commonly found in stomachs resected for cancer. Aschoff sinuses were rare at a distance from the tumor. Defects in the muscularis mucosæ at a distance from the tumor could not be found, but on the other hand, the small cells of the superficially spreading type of carcinoma seems to drop right through an apparently intact muscularis mucosæ.

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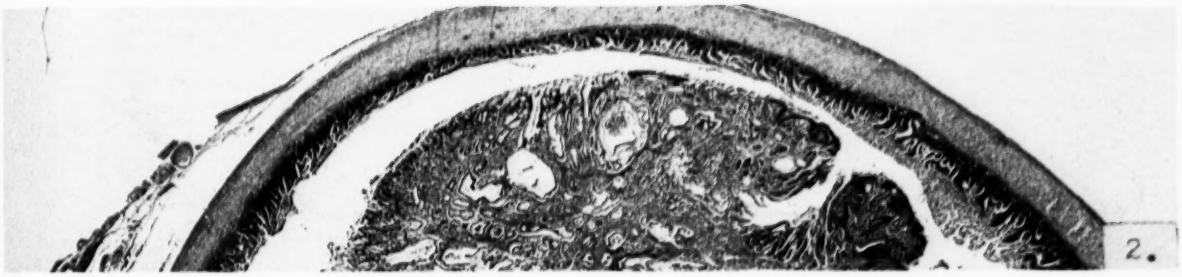
DESCRIPTION OF FIGURES 1 TO 4

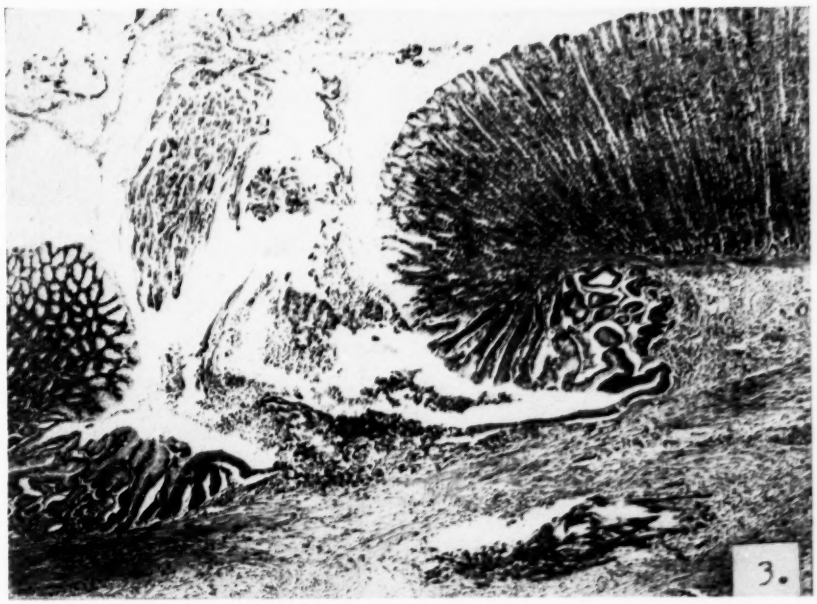
Fig. 1.—Methylcholanthrene thread, 515 days. The adenocarcinoma is breaking through wall of stomach on the right and large metastatic nodule of adenocarcinoma is present. Normal mucosa seen on extreme left and right.

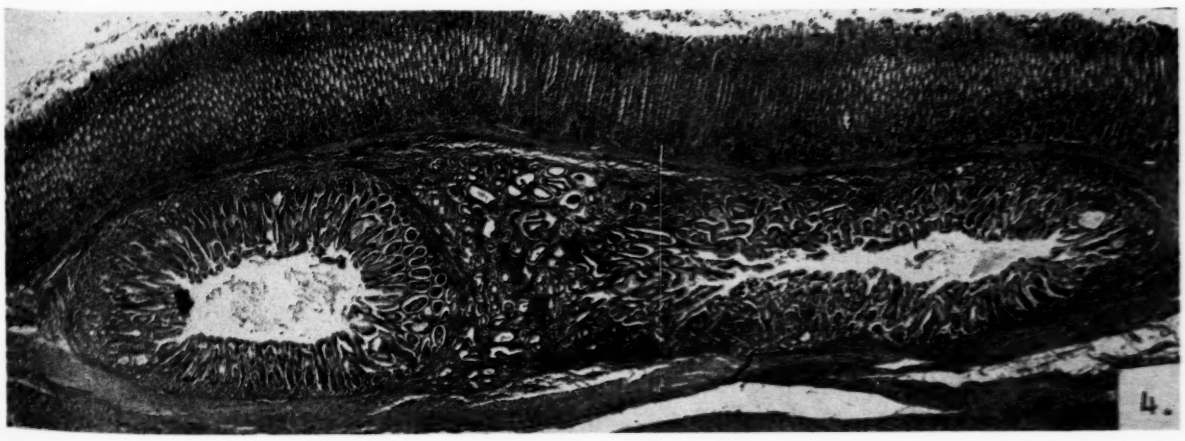
Fig. 2.—Methylcholanthrene thread, 460 days. Adenoma (?) or adenocarcinoma fills the entire lumen of the stomach. Only one-fifth of circumference shown.

Fig. 3.—Methylcholanthrene thread, 18 days, ulcerating away. On either side of defect may be seen epithelial cells that have extended beneath the muscularis mucosæ and are traveling across base of the defect in single layer. The transition from mucosa consisting of 3 types of cells to epithelium composed only of mucous cells beneath the muscularis mucosæ may be seen on the right.

Fig. 4.—Sinus tracts, four weeks old, made by methylcholanthrene thread. The character of the epithelium lining the sinus may be compared with that of mucosa. Tract is double because thread was double at this point. Thread removed by microtome.







FIGS. 1-4

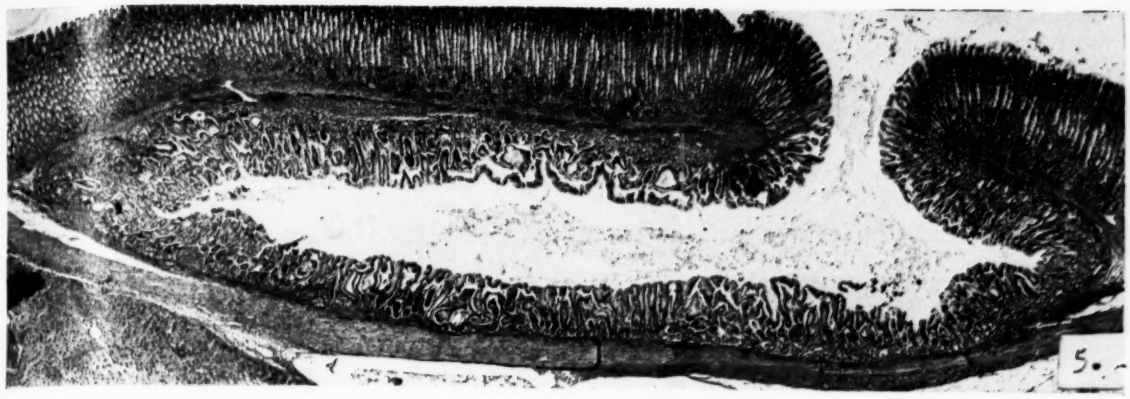
DESCRIPTION OF FIGURES 5 TO 8

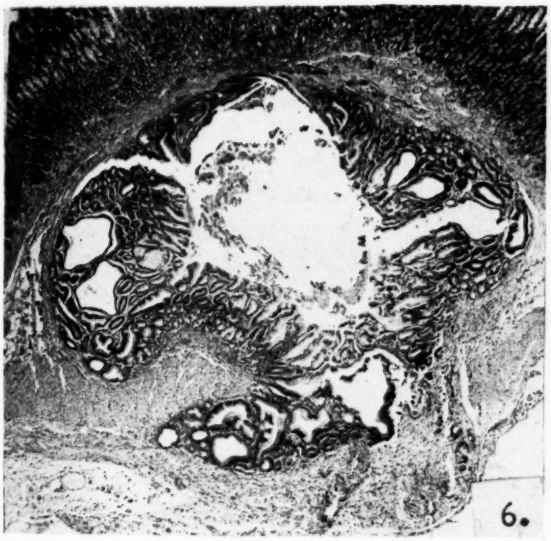
FIG. 5.—Cleft, 4 weeks old, made by methylcholanthrene thread. The area of ulceration through the mucosa can be seen as well as the transition in the epithelium.

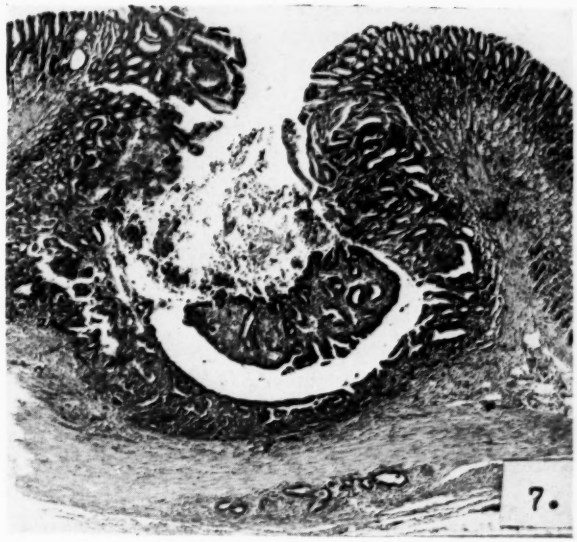
FIG. 6.—Methylcholanthrene thread sinus, 50 days. Note absence of reticulin barrier along bottom and irregularity of the growth of the epithelial cells.

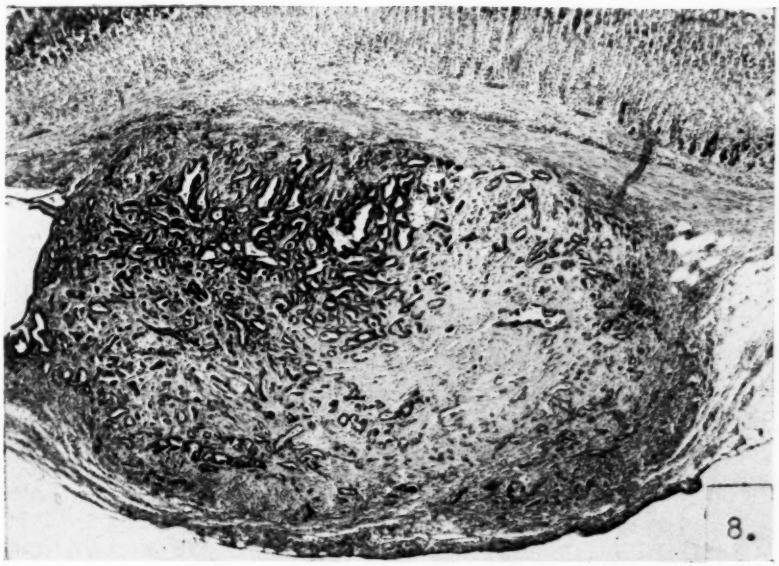
FIG. 7.—Methylcholanthrene thread, 60 days. Epithelial cells in cleft beginning regeneration. Note papillomatous growth has been cut across in center of cleft. Mouth of cleft is beginning to separate and the epithelium lining the wall has a more disordered growth than is seen in Fig. 5. In some areas they are growing in sheets. The reticulin capsule is not as well demarcated as usual around a cleft.

FIG. 8.—Methylcholanthrene thread, 200 days. Dense connective tissue surrounding numerous acini. On left is large cystic structure, partially cut off. Whether these epithelial cells are continuing their growth in spite of "scirrhous" connective tissue is not known.









FIGS. 5–8.

Tumor Immunity in Mice, Induced with Lyophilized Tissue, as Influenced by Tumor Strain, Host Strain, Source of Tissue, and Dosage*

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When tumors originating in one inbred strain of mice are transplanted into a second inbred strain (homoiotransplants) they usually show either no growth, or temporary growth followed by complete regression. The maximum size attained varies greatly according to the tumor and host strain employed, but for any one combination of tumor and host strain it is often quite uniform. Particularly is this true within any one group of animals inoculated on the same day with tissue from the same tumor transplant generation.

In previous experiments (2), mice of the recipient strain were given a series of injections of lyophilized (frozen-dried) tumor tissue, and inoculated with the living tumor about 10 days after the last injection. The results varied depending on the combination of tumor and host strain employed. Where the tumor was myeloid leukemia C1498 (C57 black origin) and the host strain the C57 leaden, the prior injections resulted in almost complete inhibition of tumor growth. In contrast to this, if the tumor was A strain carcinoma 15091a and the host strain the C57 brown, the prior injections changed the small, temporary growths that characterized the control into large tumors that killed 100 per cent of the brown hosts. Similar results were obtained with 15091a in C57 black and B alb C mice, though in the latter case the stimulation was less pronounced.

In these and subsequent experiments there have proved to be at least four significant variables: (a) the tumor used as lyophilized tissue; (b) the tumor used as fresh tissue; (c) the host strain; (d) the dosage of lyophilized tissue. At this laboratory there are at least 10 readily available inbred strains of mice and more than 20 transplantable tumors. Thus an enormous number of combinations is possible. Many combinations have been tried, though only a fraction of the total possible number. In several cases injections of lyophilized normal tissue have also been tested.

MATERIALS AND METHODS

Seven different strains of mice were used as hosts for the transplants, namely, A, B alb C, C57 black subline 6, C57 brown subline a, C57 brown subline cd, C57 leaden, and C58. All these strains have been inbred by brother $\times$ sister matings for many generations. The A and B alb C strains both have "Bagg albino" ancestry and hence are somewhat related. The C57 strains are also all related and can be traced back to a common ancestor with the C58 strain. These relationships are reflected in the behavior of transplants.

The tumors employed were 15091a (mammary gland spindle cell carcinoma, A strain origin), C1300 (neurogenic sarcoma, A strain origin), C1498 (myeloid leukemia, C57 black origin), E0771 (mammary carcinoma, C57 black origin), L946[1] (fibrosarcoma, originally a bone tumor, C57 black origin), C617 (mammary carcinoma, C57 brown^{cd} origin), C954 (hepatoma, C57 leaden origin), dbrB (mammary carcinoma, dba^{1} origin), P1534 (lymphoid leukemia, dba^{2} origin).

The method of preparing lyophilized tissue has been previously described (2). Injections were given intraperitoneally with the lyophilized tissue suspended in saline or Locke's solution.

The mice were inoculated with "healthy" living tumor by the trocar method 8 to 14 days after the last injection (unless otherwise indicated by a footnote in the tables), a small piece of tissue being implanted subcutaneously in the region of the right axilla. The same lot of tissue was used to inoculate both test and control animals.

Inoculated mice were examined twice weekly,

* Aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and by a grant to the Roscoe B. Jackson Memorial Laboratory from the National Cancer Institute.

[1] Referred to in several publications as L946AII. Since tumors L946AI, L946III, L946AIV and L946B are no longer carried, we shall refer to L946AII as simply L946.

except in the later stages of tumor growth when occasionally less frequent examinations were used, and the outline of each tumor sketched on paper ruled into one half inch squares. No instrumental measurements of the tumors were taken, but all observations were made by only one of the authors so that such variation as might be introduced by different observers was ruled out. When carried out by an experienced person an accurate measure of tumor size is provided by this method. It is to be noted that the range in tumor size is enormous. The growths vary from lumps that are barely detectable to masses covering the whole side of the mouse.

For presentation of the data it was deemed necessary to have some condensed method of indicating the amount of tumor growth in a given group of experimental or control mice. After trying several procedures, one was finally adopted giving a value which we have called the "maximum mean tumor size." The rules followed in calculating this are given below.

1. Select by inspection of drawings the day on which the drawings of tumors of the given group of animals, either test or control, have the largest mean area. If two days show approximately the same mean area, select the day on which the size is most uniform. It should be noted that if, as often happens, test and control groups reach maximum size on different days, then these different days are selected.

2. In no case select a day later than 20 days after inoculation of the living tumor, or a day following the death from tumor growth, of any of the animals. (A few cases in which this rule was not followed are indicated in footnotes to the tables.)

3. Measure the area in sq. cm. of each drawing on the selected day. Count mice negative on the selected day as having tumors of size 0.

4. Calculate the mean area for the given group of animals on the selected day.

This method gives a value for the group in question which may vary from 0.01 sq. cm. to as much as 11 or 12 sq. cm. Test and control groups are compared statistically by calculating the value of P (1).

Another statistic given in the tables is the per cent of mice killed by the tumor.

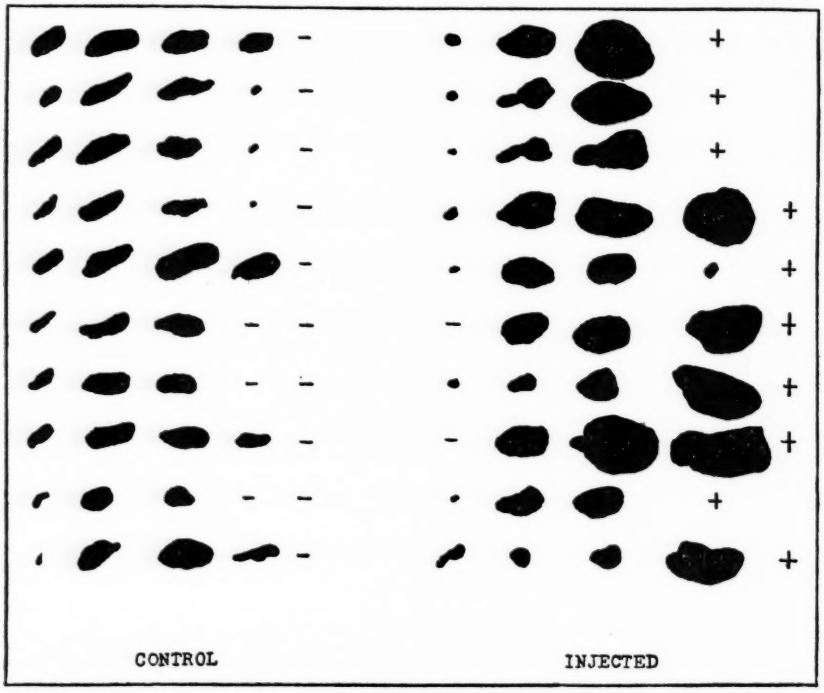

FIG. 1.—Effect of injecting C57 brown[a] mice with lyophilized L946 (C57 black origin) prior to inoculations of the living tumor. Each horizontal line represents one mouse, each vertical line a successive observation. Observations selected for figure, for both control and injected mice, were those of 7/18, 7/25, 8/1, 8/15, and 10/15. Treated mice received 7 injections of 5 mgm. each at weekly intervals, fresh L946 inoculated 13 days after the last injection. (Experiment 18, Table III.)

RESULTS

The results are summarized in Tables I to VI and Figs. 1 to 3. Their most striking feature is the diversity of effect resulting from different combina-

tions of tumor and host stock, whereas any one combination of tumor and stock, with certain specific exceptions, has given uniform and reproducible results. Despite this diversity of results, certain uniformities can be distinguished.

Mammary carcinoma 15091a, A strain origin, regularly responds to prior injections of its own lyophilized tissue by enhanced growth (Table I). This effect is most pronounced when C57 browns are employed as hosts. There are two sublines of this strain, the "a" and the "cd." These are closely similar, and assuming that they could be used interchangeably we did not at first always keep a record of the subline used. It now appears that the "a" subline may respond a little more strongly than the "cd." Tumor 15091a regularly grows temporarily and then regresses in untreated C57 brown mice. Among 93 mice used as controls there was only one exception to this rule, this one mouse being killed by the tumor. When prior injections of the lyophilized tumor were given, on the other hand, all or nearly all of the mice succumbed with large tumor masses. In early experiments, 21 injections of 5 mgm. each were used. This large number of injections, however, has proved to be unnecessary. In one lot of 10 mice (Experiment 38), subline unknown, 2 injections of 5 mgm. each resulted in 100 per cent mortality, and a total dose as low as 2.4 mgm. given in 2 injections (Experiment 79) has produced a demonstrable effect in C57 br^{a}s. There are, however, occasionally some survivors when only 2 injections are used. The possible difference in susceptibility between the two sublines is best indicated by experiment 79, where 2 injections of 1.2 mgm. each in subline a produced about the same stimulation, and 3.6 mgm. in subline a produced more stimulation, than 2 injections of 10.8 mgm. each in the brown^{cd}s.

When the C57 black strain is used as host instead of the C57 brown, the response is again one of stimulation, but less clear-cut and more variable. In two experiments 100 per cent of the treated animals died, but in other experiments the mortality has ranged as low as 16.7 per cent. The data in Table I indicate that mortality falls off with decreasing dosage and number of injections, but that other factors of an unknown nature may also be involved.

The C57 leaden and the B alb C strains also respond by enhanced growth of 15091a when given prior injections of the lyophilized tumor tissue. In an experiment (No. 83),[2] incomplete but fairly advanced, 15091a showed little or no stimulation in mice of strain C58.

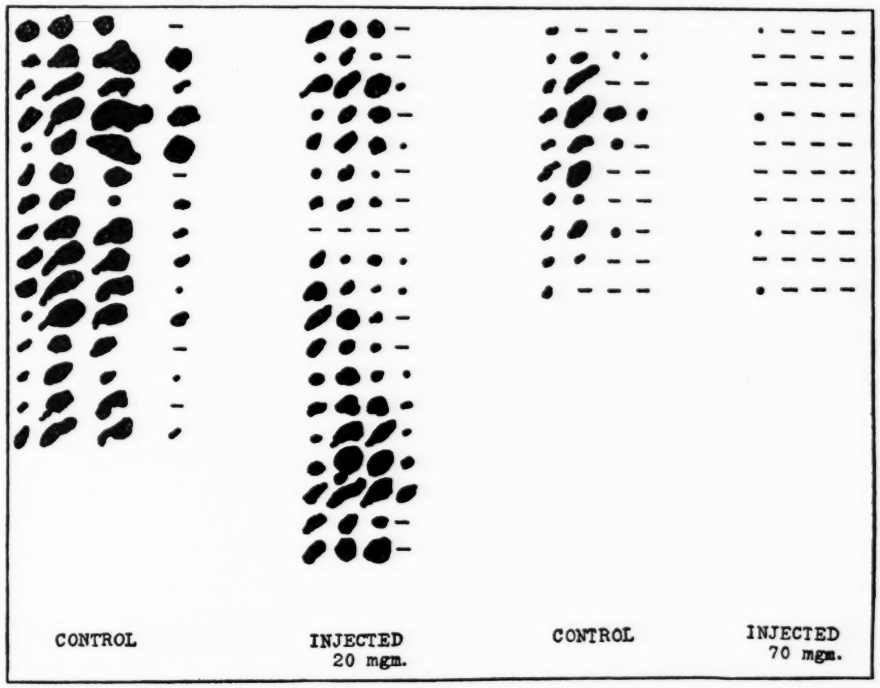

FIG. 2.—Effect of injecting B alb C mice with lyophilized L946 prior to inoculation of the living tumor. Note that whereas stimulation was produced in the C57 brown^{a} mice (Fig. 1), inhibition resulted in the B alb C strain. The larger dosage was the more effective. (Experiments 34 and 9, Table III.)

[2]Data from this experiment were lost by fire and hence are not included in the tables.

TABLE I: GROWTH OF 15091A (MAMMARY CARCINOMA, A ORIGIN) AS INFLUENCED BY HOST STRAIN, AND SOURCE AND DOSAGE OF LYOPHILIZED TISSUE

Lyophilized tissue				Control			Injected				
Source	No. of mgm. per injection	No. of injections	Exper. No.	No. of mice	% +	Max. mean tumor size	No. of mice	% +	Max. mean tumor size	Dif.	P
Host strain B alb C											
15901a	5	21	0a	20	5.0	0.10	19	58	3.95	+3.85	<.01
dbrB	5	4	33	6	0	0.17	10	20	0.06	−0.11	.2
Host strain C57 bl											
15091a	5	21	0a	23	4.3	0.94	18	100	8.39	+7.45	<.01
15091a	5	4	24	6	0	0.66	12	91.5	5.54	+4.88	<.01
15091a	5	4	50	10	30	0.50	10	100	4.69	+4.19	<.01
15091a	5	3	43	12	0	0.36	12	16.7	0.80	+0.44	.2
15091a	10	2	72	10	0	0.06	10	40	0.13	+0.07	.05
15091a*	10	2	60	9	0	1.20	10	60	1.15	−0.05	.9
15091a	20	1	60				10	60	1.72	+0.52	.2
15091a	10	1	60				10	70	1.37	+0.17	.6
15091a	3	1	60				10	30	1.77	+0.57	.2
C1300	5	4	25	6	0	0.13	12	50	3.55	+3.42	.05
C1300	10	2	37	10	0	0.77	20	30	1.07	+0.30	.2
P1534	5	4	21	5	0	0.40	10	90	5.48	+5.08	<.01
dbrB	5	4	20	5	0	0.20	11	18.2	0.55	+0.35	.2
C617	5	4	22	6	0	0.15	12	0	0.16	+0.01	.9
C954	5	4	28	5	0	0.06	9	0	0.03	−0.03	.5
Host strain C57 br[a]											
15091a	5	21	0a	20	0	0.62	18	100	6.55	+5.93	<.01
15091a	5	21	0b	7	14.3	2.36	10	100	7.35	+4.99	<.01
15091a	10	2	62	8	0	0.47	10	90	4.18	+3.71	<.01
15091a†	3.6	2	79	10	0	0.94	10	70	2.35	+1.41	.1
15091a†	1.2	2	79				10	50	3.68	+2.74	.04
Host strain C57 br[cd]											
15091a	5	4	2	8	0	0.49	5	100	9.47	+8.98	<.01
15091a	10	2	2				5	100	8.27	+7.78	<.01
15091a	10	2	65	10	0	0.43	10	70	4.26	+3.83	<.01
15091a	10	2	77	10	0	0.32	10	60	3.48	+3.16	<.01
15091a†	10.8	2	79				10	50	3.72	+2.78	.1
Host strain C57 br?											
15091a	5	2	38	10	0	0.78	10	100	6.40	+5.62	<.01
15091a	5–10	7	14	10	0	1.05	9	100	10.39	+9.34	<.01
C1300	5–10	7	14				10	100	11.05	+10.00	<.01
C1498	5–10	7	14				10	90	8.11	+7.06	<.01
C57 br. emb.	5–10	7	14				9	33.3	2.77	+1.72	.2
A embryo	5–10	7	14				10	20	2.55	+1.50	.1
Bovine plasma	5–10	7	14				8	0	2.13	+1.08	.3
Host strain C57 ln											
15091a	5	4	46	15	0	0.73	10	50	6.44	+5.71	<.01
L946	5	7	18	10	10	0.72	10	70	5.68	+4.96	<.01

NOTE: Under the heading "%+" is given the percentage of mice actually dying from the tumor.

* Lyophilized 15091a of "lot 8." Some of the tumors to provide this lyophilized tissue were infected, though all evidently infected tissue was discarded.

† Lyophilized 15091a prepared by a special procedure that will be described in a later paper. Experiment 79 is of interest because it is the only one in which C57 br[a] and C57 br[cd] mice were used simultaneously. Only one group of controls was run, consisting of ten C57 brown[a].

The six strains that have been tested with this tumor may be arranged tentatively in descending scale according to how potently they show the enhancing effect, as follows: C57 brown[a], C57 brown[cd], C57 leaden, C57 black, B alb C, C58.

Besides the tests of 15091a preceded by injections of its own lyophilized tissue, tests were made of the effect on 15091a of other lyophilized tissues. The results (Table I) show strong cross reactions with tumors C1300 (A origin), C1498 (C57 black origin), L946 (C57 black origin) and P1534 (dba origin). On the other hand there was little cross reaction with dbrB (dba origin) and none with C617 (C57 brown origin) or C954 (C57 leaden origin). Lyophilized embryos (A and C57 brown) produced slight stimulation of 15091a in C57 brown hosts (subline of brown embryo and host unknown). Dried bovine plasma was without significant effect.

Neurogenic sarcoma C1300 (A origin) tested in C57 black hosts did not respond to prior injections of its own lyophilized tissue or of lyophilized

15091a (Table II). This tumor gives particularly small masses in foreign strains and seemingly has a low growth potential.

Fibrosarcoma L946 (C57 black origin) is interesting in that homoiotransplants respond to prior injections of lyophilized L946 by inhibited growth where the hosts are B alb Cs, but by stimulated growth where they are C57 browns (Table III and Fig. 1). The effect is undoubtedly significant in each case ($P<0.01$). The inhibiting effect has been confirmed by repetition.

Mammary carcinoma E0771 (C57 black origin) responds to injections similarly to L946, but with interesting differences as shown in Table IV. The inhibiting effect with L946 in the B alb Cs is partly replaced by a stimulating effect with E0771, but that it is still present is indicated by the number of mice in the treated group already negative at the second observation (Fig. 3). Two dosages were used, 3 mgm. per injection and 15 mgm. per injection; the latter tended to emphasize the stimulating effect. Curiously enough E0771 shows no stimulation in the C57 brown^{cd} strain (Experiment 80), though tumors 15091a and L946 in brown hosts respond to prior injections of their own lyophilized tissue by potent stimulation. This experiment should be repeated, but that the result is not accidental is indicated by experiment 67, run simul-

TABLE II: LACK OF INFLUENCE OF PRIOR INJECTIONS OF LYOPHILIZED TISSUE ON GROWTH OF C1300 (NEUROGENIC SARCOMA, A ORIGIN) IN C57 BLACK MICE

Lyophilized Tissue				Control			Injected				
Source	No. of mgm. per injection	No. of injections	Exper. No.	No. of mice	% +	Max. mean tumor size	No. of mice	% +	Max. mean tumor size	Dif.	P
Host strain C57 bl											
C1300	5	4	25	3	0	0.07	12	0	0.03	−0.04	.3
C1300	10	2	37	10	0	0.01	20	0	0.02	+0.01	.3
15091a	5	4	24	4	0	0	10	0	0.01	+0.01	.5

TABLE III: GROWTH OF L946 (FIBROSARCOMA, C57 BLACK ORIGIN) AS INFLUENCED BY HOST STRAIN AND SOURCE AND DOSAGE OF LYOPHILIZED TISSUE

Lyophilized Tissue				Control			Injected				
Source	No. of mgm. per injection	No. of injections	Exper. No.	No. of mice	% +	Max. mean tumor size	No. of mice	% +	Max. mean tumor size	Dif.	P
Host strain B alb C											
L946	5	4	34	20	0	1.07	20	0	0.52	−0.55	.02
L946	10	7	9	10	0	0.45	10*	0	0	−0.45	<.01
dbrB	5	4	33	8	0	1.30	10	10	0.70	−0.60	.02
Host strain C57 br^{a}											
L946	5	7	18	10	0	1.32	10	100	4.60	+3.28	<.01
L946	15	21	18				9	100	5.98	+4.66	<.01
Host strain C57 br^{cd}											
L946	5	7	18	9	11.1	1.46	10	70	3.11	+1.65	.1
Host strain C57 ln											
L946	5–10	7	16	5	100	4.16	10	100	6.00	+1.84	.1
L946	15	7	18	10	90	3.73	10	100	7.67	+3.94	.05

*Inoculated 15 days after the last injection.

TABLE IV: GROWTH OF E0771 (MAMMARY CARCINOMA, C57 BLACK ORIGIN) AS INFLUENCED BY HOST STRAIN AND DOSAGE OF LYOPHILIZED TISSUE

Lyophilized Tissue				Control			Injected				
Source	No. of mgm. per injection	No. of injections	Exper. No.	No. of mice	% +	Max. mean tumor size	No. of mice	% +	Max. mean tumor size	Dif.	P
Host strain B alb C											
E0771	3	7	19	12	0	0.42	12	25.0	0.17	−0.25	<.01
E0771	15	7	19				12	66.7	5.94*	+5.52	<.01
E0771	10	7	9	10	0	0.44	9	77.8	3.90**	+3.46	.01
Host strain C57 br^{cd}											
E0771	10	2	80††	10	0	0.13	10	0	0.25	+0.12	.1
Host strain C57 ln											
E0771	5–10	7	16	5	60	4.25	10	80	3.26	−0.99	.7
L946	5	7	18	8	100	12.99	10	100	9.82	−3.17	.1
Host strain C58											
E0771	5	4	54	14	14.3	0.24	15†	100	4.97***	+4.73	<.01
E0771	10	2	67††	10	0	0.13	10	70	2.31	+2.18	<.01

†Inoculated 15 days after the last injection.
††Experiments 80 and 67 were run simultaneously.
*36 days after inoculation.
**34 days after inoculation.
***24 days after inoculation.

taneously with experiment 80, in which E0771 in C58 strain mice showed stimulation. This latter combination had been tried previously with similar results, 4 injections of 5 mgm. each leading to large growths and 100 per cent mortality in fifteen C58 mice. The 14 controls showed unusually small masses, though 2 of them, after apparently becoming negative, later developed fatal tumors at the site of inoculation.

The C58 strain promises to be particularly interesting. Though giving enormous stimulation when tested with E0771, it gave little or no stimulation when tested with 15091a (Experiment 83).[2] Prior injections of lyophilized 15091a slightly *inhibited* L946 in C58 mice.

Myeloid leukemia C1498 (C57 black origin) has proved to be the best tumor for demonstrating inhibition (Table V). Its growth in mice of the A, B alb C, and C57 leaden strains is more or less completely inhibited by prior injections of its own lyophilized tissue. Inhibition may also occur in the C57 brown[cd] strain, but there is a suggestion of stimulation in C57 brown[a] mice. In the C57 leaden stock, which has been used most extensively as host, there is evidence as to the extent of the inhibition as a function of dosage and number of injections. At least 4 injections of 5 mgm. each appear to be necessary to produce unquestionably significant ($P<0.01$) inhibition as measured by maximum mean tumor size, though with only 2 injections all mice were protected from progressive tumor growth whereas 2 of 10 controls were killed by progressively growing tumors. The inhibition was most pronounced after 21 injections of 5 mgm. each, distributed over a period of 7 weeks.

Some tests have been made as to cross reactions of C1498, the lyophilized tissue coming from other sources. These show that lyophilized dbrB and L946 inhibit C1498 in B alb Cs, that L946 inhibits in C57 leadens but probably stimulates in C57 brown[a] mice, that lyophilized embryos cause possible stimulation in C57 leadens.

Lymphoid leukemia P1534 (dba[2] origin) is the only tumor which has given conspicuously incon-

TABLE V: GROWTH OF C1498 (LEUKEMIA, C57 BLACK ORIGIN) AS INFLUENCED BY HOST STRAIN AND SOURCE AND DOSAGE OF LYOPHILIZED TISSUE

Lyophilized Tissue				Control			Injected				
Source	No. of mgm. per injection	No. of injections	Exper. No.	No. of mice	% +	Max. mean tumor size	No. of mice	% +	Max. mean tumor size	Dif.	P
Host strain A											
C1498	10	7	6	5	0	0.73	8	0	0.04	−0.69	<.01
C1498	5	4	35	14	0	0.43	20	0	0.03	−0.40	<.01
C1498	5	4	82	9	0	0.88	10	0	0.12	−0.76	<.01
Host strain B alb C											
C1498	5	4	48	15	0	0.27	15	0	0.01	−0.26	<.01
C1498	5	4	82	10	0	0.54	10	0	0.03	−0.51	<.01
dbrB	5	4	33	18	0	0.40	19	5.3	0.08	−0.32	<.01
L946	5	4	34	10	0	0.59	9	0	0.03	−0.56	<.01
Host strain C57 br[a]											
C1498	5	4	49	15	0	0.23	11	0	0.43	+0.20	.5
L946	5	7	18	9	0	0.88	10	30	1.57	+0.69	.1
L946	15	4	36	11	0	1.47	10*	70	4.30	+2.83	.02
Host strain C57 br[cd]											
C1498	5	4	49	5	0	0.04	5	0	0.06	+0.02	.8
C1498	5	4	63	10**	0	0.53	10**	0	0.02	−0.51	<.01
Host strain C57 ln											
C1498	5	21	0a	20	5	2.02	18	0	0.08	−1.94	<.01
C1498	5	4	49	15	33.3	1.15	15	0	0.30	−0.85	<.01
C1498	15	4	64	10	20	1.20	10	0	0.36	−0.84	<.01
C1498	5	4	64				10	0	0.35	−0.85	<.01
C1498	10	2	64				10	0	0.61	−0.59	.02
C1498	5	2	64				10	0	1.11	−0.09	.8
C1498	5–10	7	11	8	0	0.98	9	0	0.06	−0.92	<.01
15091a	5–10	7	11				9	11.1	0.34	−0.64	.05
C57 ln emb	5–10	7	11				10	10	1.49	+0.51	.2
C57 bl emb	5–10	7	11				10	30	1.39	+0.41	.3
L946	5–10	7	16	10	0	0.65	9	11.1	0.19	−0.46	<.01
E0771	5–10	7	16				6	66.7	5.53	+4.88	<.01
E0771	10	2	71	10	60†	1.94	10	30	0.30	−1.64	.05
15091a	10	2	71				10	80	2.49	+0.55	.6

* Mice infected.

** Inoculated 18 days after last injection.

† C1498 has shown increasing virulance in C57 in mice. In experiments more recent than No. 71 it has killed 70% of the control mice, though treated mice are completely protected.

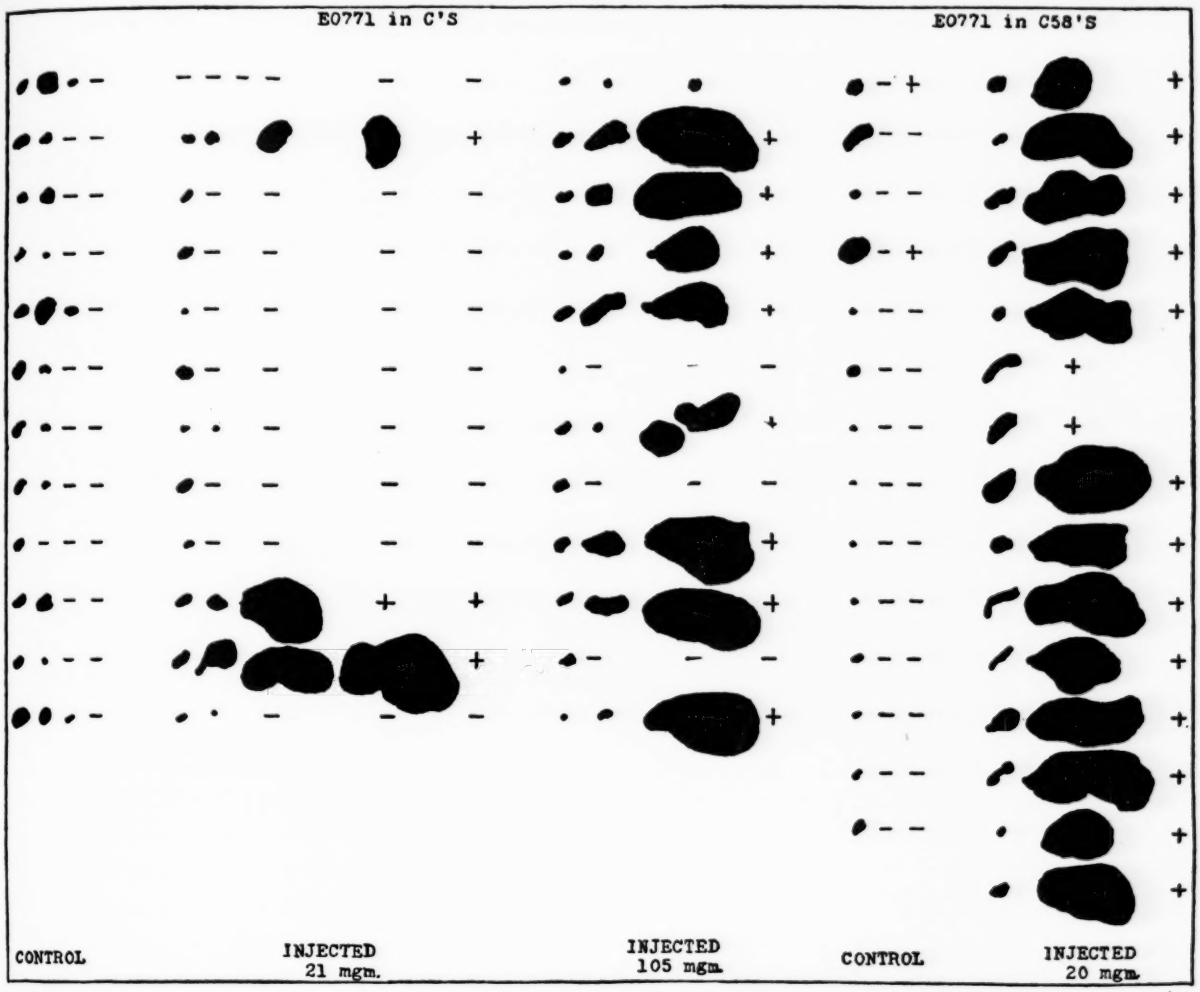

FIG. 3.—Comparison of effect of prior injections of lyophilized E0771 (C57 black origin) into B alb C (= C) strain and into C58 strain mice. In the former case there was a suggestion that inhibition occurred in some mice, stimulation in others, particularly with the smaller dosage. In the latter case there was potent stimulation, all injected mice succumbing though the dosage is only 20 mgm. Tumor E0771, like tumor L946 (Figs. 1 and 2) evidently contains both inhibiting and stimulating potentialities. (Experiments 19 and 54, Table IV.)

sistant results. This tumor, tested in both B alb C mice and in C57 blacks, in nearly every case has shown inhibited growth when prior injections of lyophilized tumor tissue have been given (Table VI). In two cases in C57 blacks (Experiments 21 and 31), significant inhibition was caused by prior injections of its own lyophilized tissue. Equally clear inhibition was produced by lyophilized dbrB (also a dba tumor). However, in experiment 59, fourteen C57 blacks injected with lyophilized P1534 gave larger tumors when inoculated with living P1534 than did 14 controls, the difference being probably significant (P=0.02). The prior injections in this case produced stimulation. It should be emphasized, however, that 100 per cent of the tumors regressed, not only in the controls but also in the treated group. The stimulation was thus not of the same order as that produced by 15091a in C57 browns, where there is 70 to 100 per cent mortality in injected mice.

It is noteworthy that the growth of P1534 in the black animals was inhibited by previous injections of 15091a (Experiments 24 and 40). Evidently 15091a has inhibiting as well as stimulating potentialities.

DISCUSSION

It would be premature to offer explanations of the fact that tumor homoiotransplants in certain host-tumor combinations are inhibited, in others stimulated, by previous injections of the frozen-dried tumor. It is in order, however, to point out that the host-tumor combination is of primary importance and that, in the experiments here reported, other

TABLE VI: GROWTH OF P1534 (LYMPHOID LEUKEMIA, DBA ORIGIN) AS INFLUENCED BY HOST STRAIN AND SOURCE OF LYOPHILIZED TISSUE

Lyophilized Tissue				Control			Injected				
Source	No. of mgm. per injection	No. of injections	Exper. No.	No. of mice	% +	Max. mean tumor size	No. of mice	% +	Max. mean tumor size	Dif.	P
Host strain B alb C											
P1534	5	4	57	15	13.3	1.65	10	40	1.12	−0.53	.05
dbrB	5	4	33	10	30	2.05	10	0	0.24	−1.81	<.01
L946	5	4	34	10	50	2.22	9	11.1	1.01	−1.21	.2
Host strain C57 bl											
P1534	5	4	21	6	0	1.33	9	0	0.22	−1.11	<.01
P1534	5	4	31	20	0	0.23	20	5	0.08	−0.15	<.01
P1534	5	4	59	14		0.48	14	0	1.10	0.62	.02
dbrB	5	4	20	Same as 21			12	0	0.15	−1.18	<.01
dbrB	5	4	32	10	0	0.40	10	0	0.09	−0.31	<.01
C1498	5	4	59a	14	0	0.48	15	0	0.47	−0.01	.9
15091a	5	4	24	5	0	0.93	10	0	0.13	−0.80	.1
15091a	5	4	40	9	0	0.35	23	0	0.14	−0.21	.01
C617	5	4	22	6	0	0.18	10	0	0.86	0.68	.04

factors cannot in any major way be responsible for these contrasting reactions.

The method of preparation of the tissue is not responsible. In early work on "tumor immunity" induced with non-living tissue (2), long storage of the tissue and other unusual procedures were used, particularly in seeking the stimulating effect. Our lyophilized (frozen-dried) tissues have been prepared by a uniform technic. Despite this fact, lyophilized C1498 produces inhibition of C1498 in C57 leaden mice, whereas lyophilized 15091a produces stimulation of 15091a in C57 browns. Although the use of a uniform method of lyophilization has ruled out method of preparation as a major factor in our experiments, it is of course quite possible that tissues prepared by different technics would give different results.

The tumor used for inoculation is not the controlling factor in determining whether stimulation or inhibition is produced. It is true that mammary carcinoma 15091a, with three exceptions of very doubtful significance, has always responded to prior injections by stimulated growth, while myeloid leukemia C1498 and lymphoid leukemia P1534 have almost always shown inhibition in treated mice. On the other hand fibrosarcoma L946 (bone origin) shows unquestionable inhibition in B alb C mice, but stimulation in injected C57 browns. Mammary carcinoma E0771 likewise appears to have ambivalent potentialities, but responds predominantly by stimulation. There is a suggestion in these results that leukemias are most apt to respond by inhibition, mammary tumors by stimulation. On the other hand leukemia C1498 is stimulated in one instance, namely, in C57 brown[a] mice injected with lyophilized L946 (Table V, Experiments 18 and 36; note particularly the percentage killed). Also prior injections of lyophilized mammary carcinoma dbrB are almost more effective in inhibiting leukemia P1534 than are injections of P1534's own lyophilized tissue (Table VI). In any case the relation of tumor type to stimulating and inhibiting effects deserves further tests.

The mice used as hosts are likewise not alone responsible. When C57 brown[a] mice have been used as hosts, tumors have always responded to prior injections by stimulated growth. The A strain, tested only with C1498, has given inhibition only. On the other hand both stimulation and inhibition have occurred in all other strains tested (B alb C, C57 black, C57 brown[cd], C57 leaden, C58).

In the special case of E0771 in B alb Cs, dosage appears to be significant in determining whether prior injections produce stimulation or inhibition (Fig. 3 and Table IV, Experiment 19). In all other instances the only significance of dosage is the obvious one that too few injections or too small amounts per injection are ineffective.

We are thus left with the conclusion that the host-tumor combination, or more precisely the host-lyophilized tissue-fresh tissue combination, is the significant factor in determining whether inhibition or stimulation is produced. That there are other undetermined factors of at least some importance is indicated by the lack of consistency in the one case of P1534 in C57 black hosts.

SUMMARY

Mice were given a series of injections of lyophilized tumor tissue, or in a few cases of lyophilized normal tissue, and inoculated with living tumor about 10 days after the last injection. Seven different inbred strains of mice (A, B alb C, C57 black[s], C57 brown[a], C57 brown[cd], C57 leaden, C58) were used as host, 9 different tumors (15091a, C1300, C1498, E0771, L946, C617, C954, dbrB,

P1534) to provide lyophilized tissue for injection or fresh tissue for inoculation, a total of 45 host-lyophilized tissue-fresh tissue combinations being tested. In the controls, the tumors with few exceptions showed the behavior typical of homoiotransplants, namely, moderate growth followed by regression. In the injected mice the results depended on the particular host-tissue combination. In some combinations growth of the tumors was almost completely inhibited, in others it was stimulated to the point where 70 to 100 per cent of the mice were killed. One tumor, L946, gave strong inhibition or stimulation according to the strain in which it was tested. In a few cross tests between different tumors there were indications of some specificity when the tumors used to provide lyophilized tissue and fresh tissue originated in different strains. Tumors originating in the same strain gave strong cross reactions. Lyophilized embryo in two tests showed slight activity, dried bovine plasma little or none. Some of the host tumor combinations have been repeated a number of times and with one exception have given consistent results.

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Effect of Cold and of Beef Spleen Extract on dbrB Mouse Tumor Cells as Shown by Growth of Transplants into dba Mice and by Cytologic Examination*

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Growth of autografts of mouse tumors previously immersed in aqueous extracts of desiccated embryo skin and of mouse placenta has been retarded and in some instances the grafts have failed to grow (15). Growth of the Bashford adenocarcinoma 63 has been inhibited in mice when the tumor was exposed to aqueous extract of mammary tissue from pregnant rabbits prior to implantation (14). Flexner-Jobling rat carcinoma and mouse sarcoma 180 were stored in the refrigerator for 2 and 5 days respectively with an aqueous extract of rat spleen. The number of takes of the rat carcinoma was reduced upon transplantation, whereas the mouse carcinoma failed to grow (16).

The present paper reports the effects of storage in the refrigerator for 1 to 7 days on tumor cells suspended in an aqueous, alcohol-precipitated beef spleen extract implanted into mice, and a cytologic study at the time of implantation. The investigation was undertaken in an effort to devise an assay method for beef spleen extract which has been useful in the treatment of basal cell epitheliomas in human beings (1, 2).

MATERIALS AND METHODS

The dbrB adenocarcinoma and dba mice were selected for these preliminary tests on the effect of storage in beef spleen extract, since this homologous, non-invasive tumor when implanted into this strain of mice uniformly grows in 100 per cent of the cases and does not undergo spontaneous regression.

Tumors from the donor animals were removed under aseptic conditions and the necrotic portions dissected away. The firm tumor tissue was rubbed through a fine wire mesh and the puree obtained was diluted 3 to 1 by volume with Ringer's solution containing beef spleen extract, and with Ringer's solution alone for the control experiments. The cell suspensions, in stoppered centrifuge tubes, were stored in the refrigerator at 5° ± 2° C.

Isotonic Ringer's solution served as the control storage medium, and the experimental solutions were Ringer's containing beef spleen extract diluted on the basis of 40, 60, 80, 100, and 112 mgm. of solids per ml. of final solution (pH 7.0). Flocculation and gelation of the tumor cell suspensions occurred in concentrations of 80 mgm. per ml. and above. The experimental solution at 78 mgm. per ml. had a tonicity 2.5 times that of isotonic Ringer's solution as determined by the depression of the freezing point so that a control experiment was made with Ringer's solution of 2.5 normal tonicity as a storage medium.

The tumor cell suspension in Ringer's solution had a pH of 6.4 which remained constant for 1 hour, but changed to 6.82 and 6.65 after 1 day and 5 days respectively. Similar suspensions in beef spleen extract diluted in Ringer's solution to 70 mgm. per ml. had pH values of 7.75, 7.30 and 7.29 at the end of 1 hour, 1 day and 5 days respectively.

On the day of inoculation, the cell suspensions were removed from the refrigerator 1 hour before use and gently rotated from time to time. Young dba male mice, 4 to 8 weeks old, were inoculated subcutaneously in the dorsal region with 0.1 ml. of the cell suspension. No perceptible local reaction to the injection occurred, although the tumor derives from its host a rich vascular supply. Once the tumors became palpable (2mm. in diameter) the latent period varying in each experiment, they increased 1 to 2 mm. in each direction each day, the nodules remaining almost free of the skin and underlying tissue. Many of the tumors attained a volume equal to or exceeding that of the host, and the animals eventually died of the tumor. There was no apparent histologic difference in the tumors derived from treated and control cells. A total of 100 mice were injected with the control tumor suspensions, 16 with suspensions stored in

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hypertonic Ringer's solution, and 182 received the suspensions in beef spleen extract of different concentrations. The suspensions were stored at 5° C. in the refrigerator for periods of 1 to 7 days.

Squash preparations from a portion of the tumor cell suspensions as well as from blocks of the tumor tissue were made at the same time as the inoculations of the control and experimental stored tissue. The fixative used was absolute alcohol and glacial acetic acid (3:1) and the preparations were stained with aceto-carmine. Measurements of the nuclear volume were made in 4 preparations of the control and of the experimentally treated tumor cells. Nuclei of 450 to 1200 cu. μ were classified as nuclei of Class II, those of 1200 to 1800 cu. μ as those of Class III according to Biesele, Poyner, and Painter (6). In a few preparations the Feulgen stain was used.

RESULTS

In vivo, control experiments.—Storage of dbrB tumor cell suspensions at 5° C. in isotonic Ringer's solution for as long as 8 days did not greatly reduce the number of tumors developing from the stored cell suspensions upon implantation into test dba mice, 94 tumors appearing in a total of 100 control mice (Fig. 1; Table I). The outstanding effect of storage at 5° C. in isotonic Ringer's solution was progressive prolongation of the normal 6 to 8 day latent period prior to the appearance of palpable tumors (Fig. 2). The values used for the graph are averages of the data presented in Table I. Storage of tumor cells in hypertonic Ringer's solution (equivalent in tonicity to a solution containing 78 mgm. per ml. of spleen extract) for periods of 1, 2, and 8 days reduced the number of tumors developing upon transplantation into mice, and greatly extended the latent period of the tumors which did develop (Figs. 1 and 2; Table I). A seasonal factor may have operated in the 2 and 8 day storage experiment with hypertonic Ringer's solution and in the control tissue, as the experiment was conducted in July and August when the latent period for the appearance of palpable tumors is usually longer than at other seasons of the year.

In vivo, spleen extract experiments.—Storage of tumor cells in 40 mgm. per ml. spleen extract for 1 to 5 days at 5° C. resulted in tumor development in 94 per cent of the 50 test animals and 100 per cent of 30 control animals (Table I). The longer

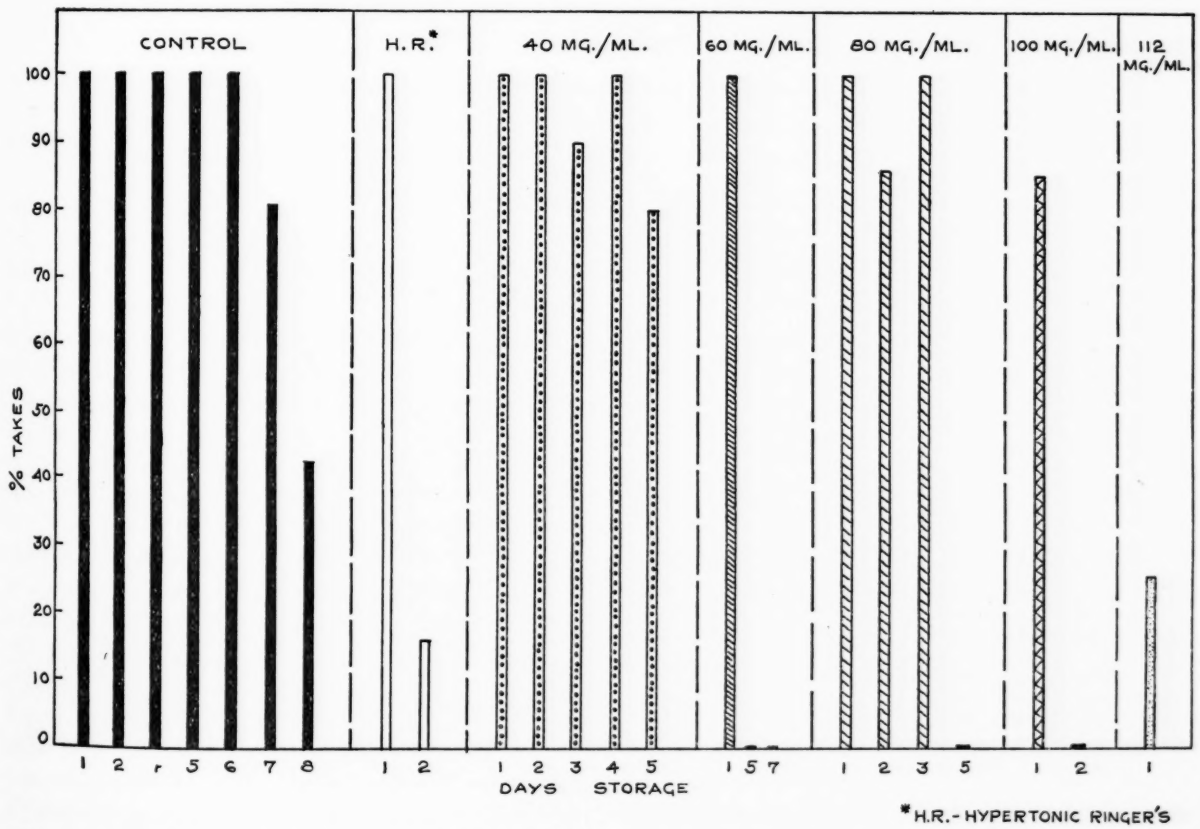


FIG. 1.—Development of dbrB tumors in dba mice receiving cell suspensions stored at 5° C. in isotonic and hypertonic Ringer's solution and beef spleen extract for 1 to 7 days.

the period of storage in 40 mgm. per ml. spleen extract the longer the latent period for tumor development, the longest being 18 and 19 days after 4 and 5 days, respectively (Fig. 2; Table I).

Storage of tumor cells in 60 mgm. per ml. of spleen extract for 1, 5, and 7 days resulted in tumor development in 10 of 30 animals as contrasted with 14 tumors in the 15 control animals, or 93 per cent takes. The latent period for the 10 tumors developing after storage for 1 day in 60 mgm. per ml. ranged from 6 to 14 days (average, 8 days). No tumors had developed by the 43rd and 41st day after storage for 5 and 7 days, respectively, in 60 mgm. per ml. spleen extract.

TABLE I: VIABILITY OF DBRB TUMOR CELLS STORED AT 5° C. IN ISOTONIC RINGER'S SOLUTION, HYPERTONIC RINGER'S SOLUTION, AND IN BEEF SPLEEN EXTRACT

Spleen extract conc.	No. test animals	No. tumor takes	Latent period, days Range	Average
		1-DAY STORAGE		
Control	6	6	6–14	10
Hypertonic Ringer's soln.	6	6	6–17	13
Control	10	10	7–10	9
40 mgm./ml.	10	10	10	10
Control	5	5	6–7	6.5
60 mgm./ml.	10	10	6–14	8
80 mgm./ml.	10	10	6–19	12
Control	10	10	6	6
100 mgm./ml.	20	17	20–36	22
112 mgm./ml.	4	1	28	28
		2-DAY STORAGE		
Control	6	6	14–23	18
Hypertonic Ringer's soln.	6	1	33	33
40 mgm./ml.	10	10	9–15	12
Control	2	2	11	11
80 mgm./ml.	3	1	19	19
80 mgm./ml.	9	9	8–10	9
Control	5	5	5–6	5.5
80 mgm./ml.	10	9	18–24	20
Control	10	10	6–14	11
100 mgm./ml.	20	None by day 56	—	—
		3-DAY STORAGE		
40 mgm./ml.	10	9	12–21	16
80 mgm./ml.	4	4	17–23	19
		4-DAY STORAGE		
Control	10	10	10–17	13
40 mgm./ml.	10	10	14–25	18
		5-DAY STORAGE		
Control	10	10	13–17	15
40 mgm./ml.	10	8	15–25	19
Control	5	5	8–12	10
60 mgm./ml.	10	None by day 43	—	—
Control	3	3	18–19	18.5
80 mgm./ml.	2	None by day 30	—	—
80 mgm./ml.	10	None by day 43	—	—
		7-DAY STORAGE		
Control	11	9	12–16	14
60 mgm./ml.	10	None by day 41	—	—
		8-DAY STORAGE		
Control	3	3	14–17	15
Control*	4	None by day 71	—	—
Hypertonic Ringer's soln.*	4	None by day 71	— —	— —

* Experiments conducted during July and August, 1947.

Storage for 1, 2, 3 and 5 days in 80 mgm. per ml. extract resulted in tumor development in 68 per cent of 48 experimental animals, as compared with 100 per cent takes in the 15 control mice. After 1, 2 and 3 days of storage of tumor cells the average latent period for tumor development in 4 groups of mice was 12 (1 day storage), 19 and 20 (2 day storage), and 19 days (3 day storage). In a fifth group of 9 animals receiving tumor cells stored for 2 days in 80 mgm. per ml. the latent period was 8 to 10 days. In this experiment the inoculum spread under the skin and produced multiple palpable nodules 10 days earlier than was the case with the other 2 groups of animals receiving cells stored for the same period of time under the same circumstances (Table I). After 5 days of storage in 80 mgm. per ml. and implantation into 12 mice, no tumors developed within 30 to 43 days of observation (Table I).

Tumor cell suspensions stored for 1 day in 100 mgm. per ml. spleen extract in normal saline produced tumors in 17 of 20 mice after a latent period of 20 to 36 days, whereas cells stored in 100 mgm. per ml. for 2 days had produced no tumors in 20 test mice at the end of 56 days. All 10 of the control animals had tumors within 6 to 14 days (average 11 days).

Of 4 mice receiving tumor cell suspensions stored for 1 day in 112 mgm. per ml. spleen extract, 1 animal developed a tumor after a latent period of 28 days.

Cytology.—Observations on the cytologic effects of storage in the refrigerator on: (a) dividing cells; (b) resting cells; and (c) nuclear volume in isotonic and hypertonic Ringer's solution, and in 40, 60, 80 and 100 mgm. per ml. spleen extract for 24 and 48 hours, and for periods as long as 7 days are successively presented.

Dividing cells. (Storage for 1 day).—Mitotic activity was progressively decreased by storage in isotonic Ringer's solution at 5° C. The proportion of 11 larger polyploid type of nuclei in 500 cells was unchanged by 24 hours of storage. Mitotic figures were rare after storage for the same period of time in hypertonic Ringer's solution, and while some were normal, usually the chromosomes were clumped at metaphase and at telophase. Storage in spleen extract (40 mgm. per ml.) for 24 hours considerably reduced the number of mitotic figures,

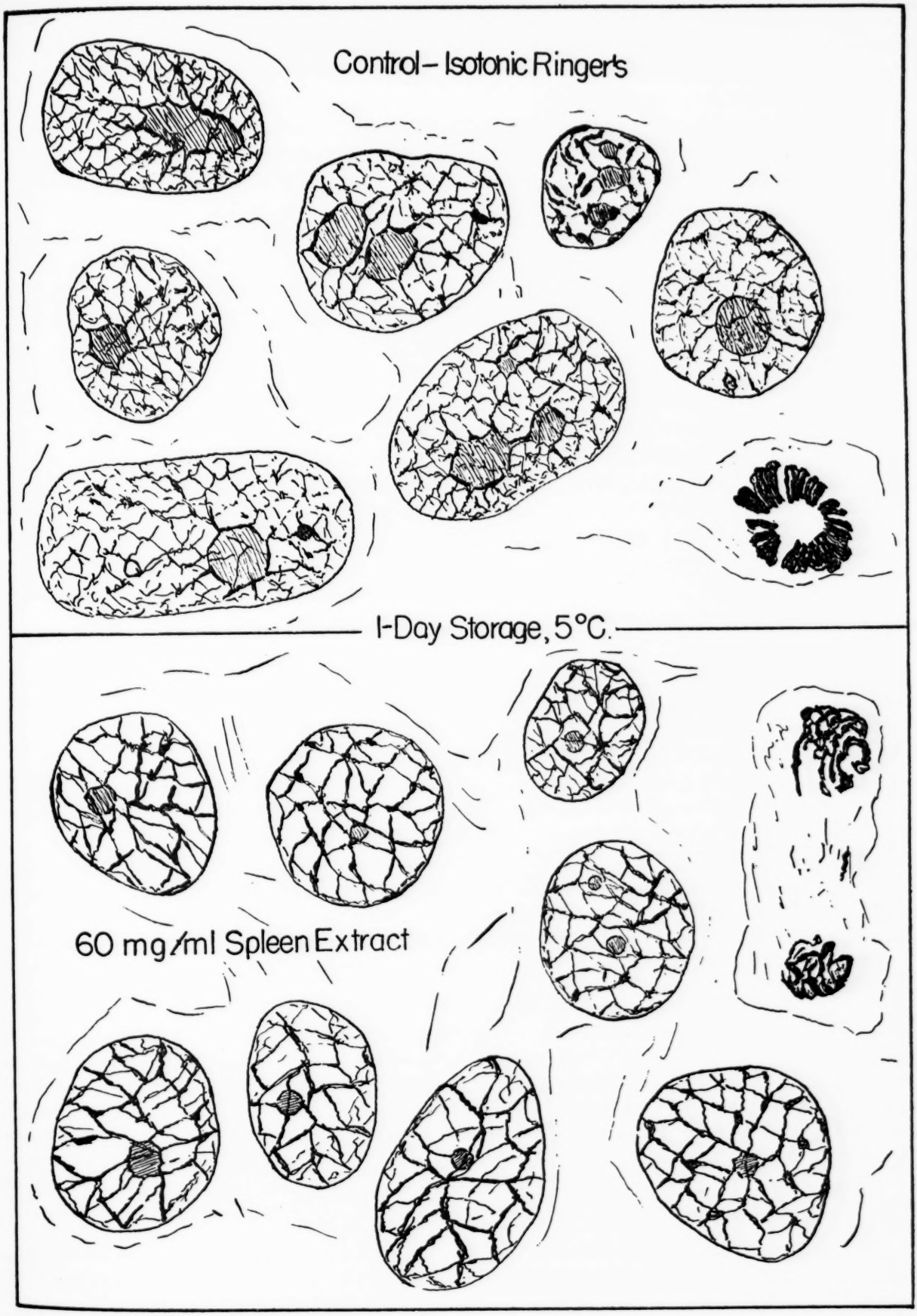


FIG. 3.—Camera lucida drawings of selected nuclei from squash preparations of suspensions of dbrB tumor cells stored at 5° C. for 24 hours in isotonic Ringer's solution and beef spleen extract (60 mgm. per ml.). Alcohol-acetic acid fixative, 3:1, and aceto-carmine stain. Mag. × 2,418.

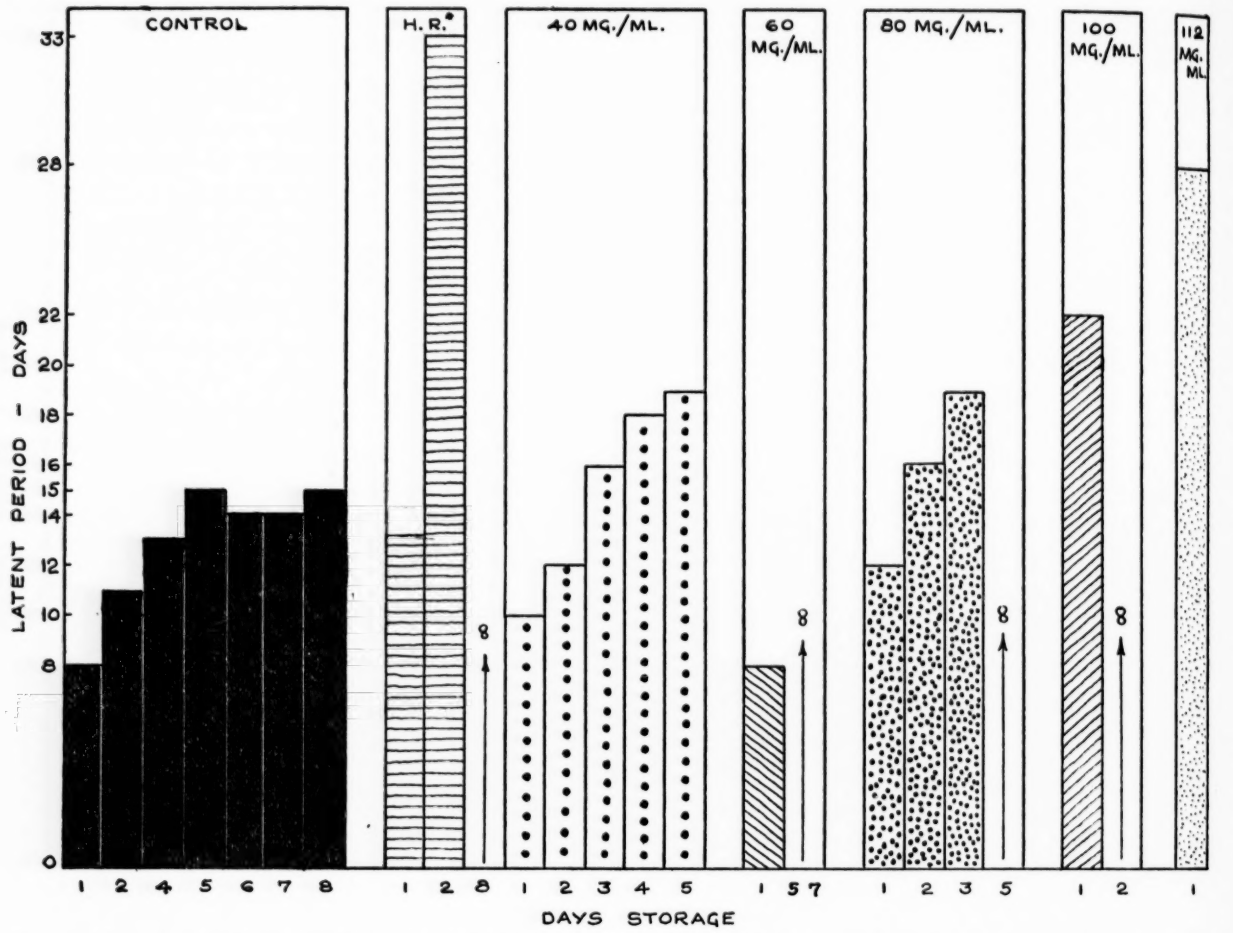


FIG. 2.—Latent period for dbrB tumor development in dba mice receiving cell suspensions stored at 5° C. in isotonic and hypertonic Ringer's solution and in beef spleen extract for 1 to 7 days.

those present being fairly normal in appearance. In 60 mgm. per ml. spleen extract for 24 hours the number of dividing cells in metaphase was but 6 per cent of the number in the control cells of the same age. The chromosomes were clumped in the few telophase figures observed (Table II). Five of 500 nuclei were of the larger polyploid type (over 1800 cu. μ in volume). Some of the cells containing clumped chromosomes appeared to have been killed in mitosis. After 24 hours of storage in 100 mgm. per ml. spleen extract in normal saline there were very few cells in mitosis and they contained clumped chromosomes.

Storage for 2–7 days.—There were no mitotic figures found in cell suspensions after 48 hours of exposure to hypertonic Ringer's solution. In cells stored in isotonic Ringer's solution for as long as 5 days there was during this interval a decrease of 72 per cent in the number of cells in metaphase (Table II). Metaphase chromosomes were clumped into a horseshoe-shaped mass at the end of this period. Occasionally a cell division in telophase was distinguishable but in most instances the chromosomes were clumped into an irregular mass. Rarely, a prophase of an endomitotic type was seen in tumor cells stored in isotonic Ringer's solution. After as long as 4 days of storage in spleen extract (40 mgm. per ml.) a cell in normal telophase was observed. With 60 mgm. per ml. of spleen extract there were very few dividing cells at all times, during the period of storage (Table II), and in these cells the chromosomes appeared abnormal or clumped.

Resting cells. (Storage for 1 day).—In isotonic

DESCRIPTION OF FIGURE 4.

FIG. 4.—Camera lucida drawings of selected nuclei from squash preparations of dbrB tumor cell suspensions stored for 5 days at 5° C. in isotonic Ringer's solution and in beef spleen extract (60 mgm. per ml.). Alcohol-acetic acid fixative, 3:1, and aceto-carmine stain. Mag. × 2,800.

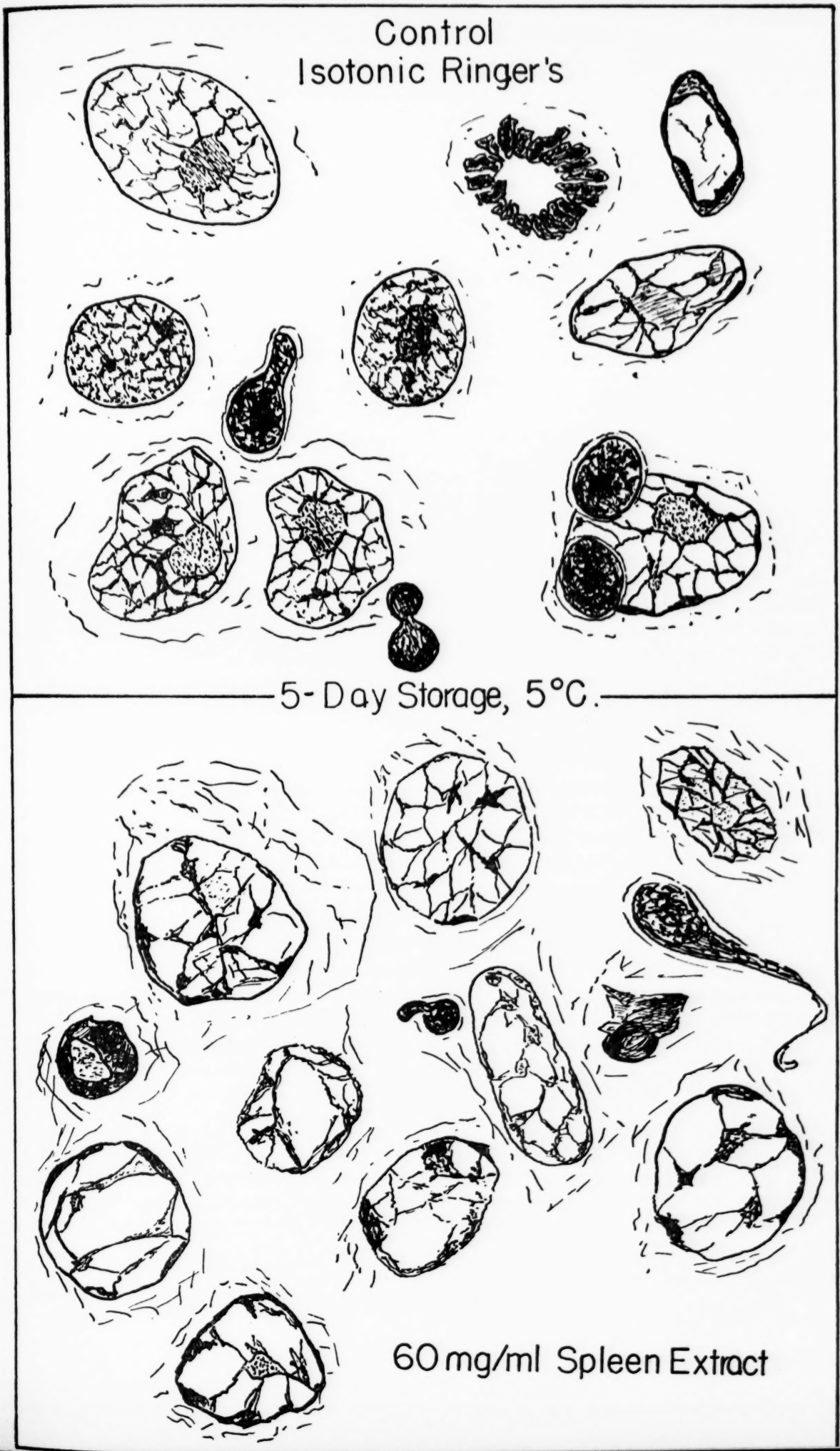

Control
Isotonic Ringer's
5-Day Storage, 5°C.
60 mg/ml Spleen Extract

TABLE II: OBSERVED PHASES OF MITOSES IN A SQUARE CENTIMETER OF SQUASH PREPARATIONS OF DBRB TUMOR CELL SUSPENSIONS PREPARED 1 HOUR AFTER REMOVAL FROM STORAGE AT 5° C. IN A CONTROL ISOTONIC RINGER'S SOLUTION AND IN 60 MGM. PER ML. BEEF SPLEEN EXTRACT. ACETOCARMINE STAIN

Days of storage	Frequency of observed mitotic phases: Metaphase		Anaphase		Telophase	
	Control	Exper.	Control	Exper.	Control	Exper.
1	50	3	5 (early)	0	10 (5 clumped)	6 (clumped)
4	25	2	5 (clumped)	0	15 (10 clumped)	0
5	12 (clumped*)	5 (pyknotic)	1 (clumped)	1 (clumped)	4 (2 clumped)	3 (pyknotic)
7	4	0	0	0	1 (pyknotic)	1

* Chromosomes abnormally adherent.

Ringer's solution at 5° C. for 24 hours, the tumor cell nuclei in interphase or resting stage contained usually a large fusion nucleolus 3–5 μ in diameter (Fig. 3), or 2 large nucleoli, and a fine weakly staining reticulum with few and inconspicuous heterochromatic or condensed segments. This type of nuclear morphology replaced that described by Biesele (5) as characteristic of malignant cells in mice in which the nuclei had multiple nucleoli and very numerous heterochromatic segments in the reticulum. There were a few smaller tumor cells of the diploid type with conspicuous heterochromatic segments. Both the nuclei and their nucleoli appeared turgid in fixed preparations.

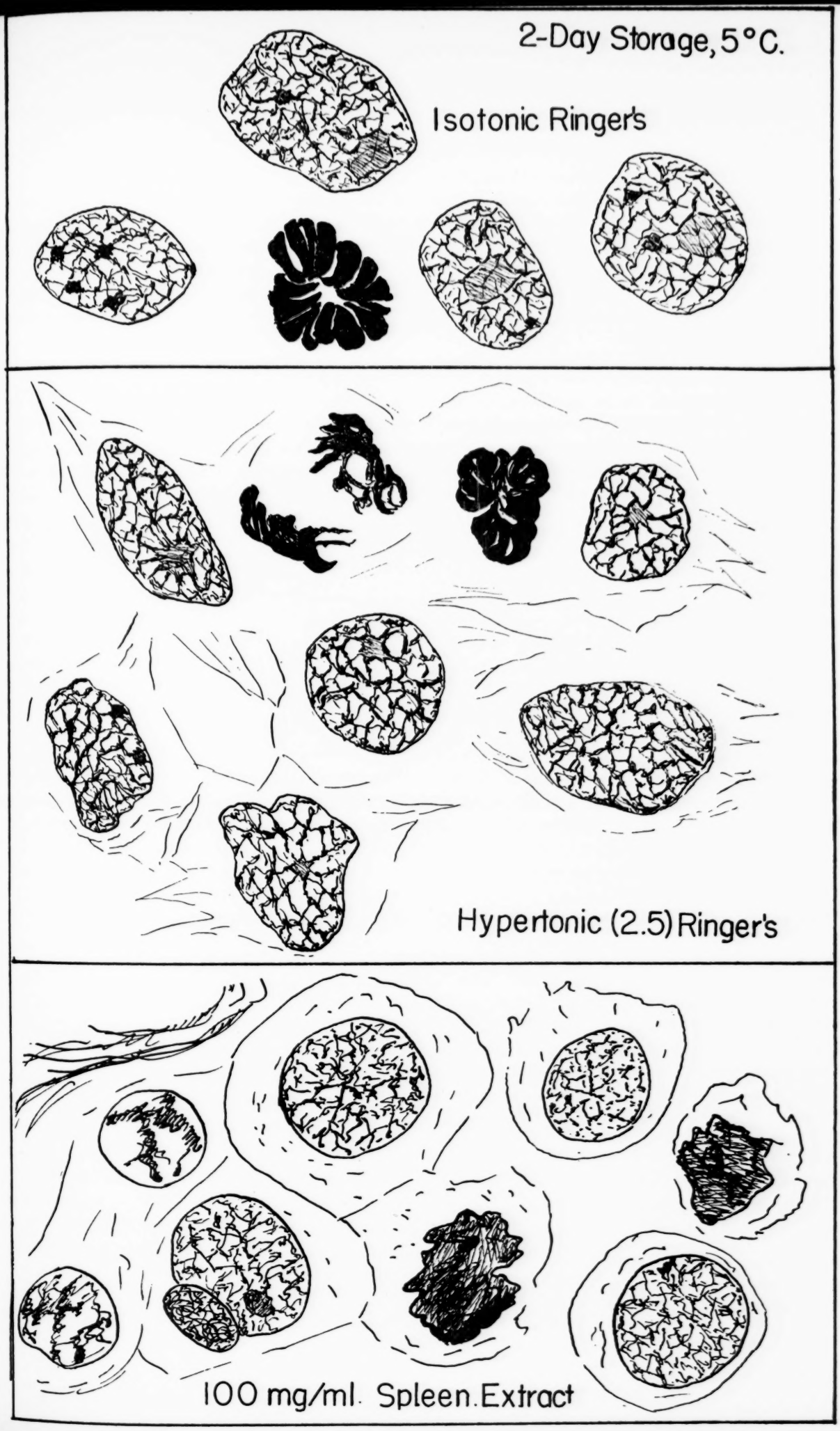
Fragments of resting tumor cells after 24 hours at 5° C. in hypertonic Ringer's solution had a denser cytoplasm than control cells in isotonic Ringer's solution; pointed, pseudopodia-like processes projected from the surface of the cells. The nuclei frequently were distorted and irregular in outline; in some the chromatin reticulum, like that of nuclei stored for the same period of time in isotonic Ringer's solution, was distinct and fine with small heterochromatic knots. In hypertonic Ringer's solution, nucleoli were present in many cells, in some instances were star-shaped, and appeared to be suspended by coarse chromatin threads inside a clear zone in the nucleus. In other cells in hypertonic Ringer's solution, the chromatin reticulum was coarse; the nucleolus when present was small, and it was sometimes absent. In fresh preparations in hypertonic Ringer's solution the nuclear membrane was indistinct. A similar appearance was described by Belar (4) in living spermatocytes of grasshoppers in hypertonic Ringer's solution (5 times that of isotonic Ringer's solution).

Storage of tumor cells in 40 mgm. per ml. spleen extract for 24 hours resulted in little alteration in the chromatin reticulum or in the nucleoli, the latter remaining 3 to 5 μ in diameter as in the control cells. In both 60 and 80 mgm. per ml. spleen extract at 5° C. for 24 hours, the nucleoli were reduced to an average of less than 2 μ in diameter (Fig. 3). In these 2 concentrations of spleen extract, the chromatin reticulum of the tumor cell nuclei was coarser than in the control cells but was regular with very small scattered, darker staining knots in some nuclei; in others the knots were absent. The cytoplasm of these cells appeared denser when stained with aceto-carmine than that of control cells stored in Ringer's solution. In fresh preparations after 24 hours of storage at 5° C. in 65 mgm. per ml., the nuclear membrane was indistinct, an appearance similar to that observed after storage in hypertonic Ringer's solution. After 24 hours at 5° C. in 100 mgm. per ml. spleen extract in normal saline, practically none of the nuclei contained nucleoli or darker staining segments on the fine regular chromatin reticulum. The cells were rounded in form. Longer periods of storage produced little additional change in the cells except for an increase in the number of pyknotic nuclei (Fig. 5).

Storage for 2 to 7 days.—After 4 and 5 days of storage in Ringer's solution, hollow empty nuclei appeared in some of the tumor cells, as well as nuclei with a coarse open reticulum and irregular patches of chromatic material on the nuclear membrane (Fig. 4). After 2 days in hypertonic Ringer's solution (at 5° C.) the reticulum remained fine and there were no normal-appearing, fusion-type nucleoli (Fig. 5). A few star-shaped structures were seen similar to those observed after 1 day of exposure to hypertonic Ringer's solution at 5° C. The contrast in nucleolar form in this and in isotonic Ringer's solution is clearly shown in Fig. 5.

DESCRIPTION OF FIGURE 5.

FIG. 5.—Camera lucida drawings of selected nuclei in squash preparations of dbrB tumor cell suspensions after 2 days of storage at 5° C. in isotonic Ringer's solution, Mag. × 2,800; in hypertonic (2.5 or the equivalent of 78 mgm. per ml. spleen extract) Ringer's solution, Mag. × 2,930; and in 100 mgm. per ml. beef spleen extract, Mag. × 3,100. Alcohol-acetic acid fixative 3:1, and aceto-carmine stain.


2-Day Storage, 5°C.
Isotonic Ringer's
Hypertonic (2.5) Ringer's
100 mg/ml. Spleen Extract

Tumor cells stored for 4 days in 40 mgm. per ml. of spleen extract resembled those cells stored for 1 day in 60 mgm. per ml. (Fig. 3), the nucleoli being reduced in size and the chromatin somewhat coarse and regular. After 5 days of storage at 5° C. in 60 mgm. per ml. of spleen extract the nuclei contained large clear vacuolar spaces (Fig. 4). The chromatin formed irregular patches on the nuclear membrane connected by a few coarse strands of chromatin. Such nuclei apparently contained no nucleoli. Occasionally there would be a nucleus with a fine chromatin reticulum showing small deeper staining segments. Some of the nuclei were shrunken and wrinkled. The cytoplasm of tumor cells stored for this period of time was more conspicuous than that of cells stored for a similar period in isotonic Ringer's solution. After 7 days of storage at 5° C. in 60 mgm. per ml. spleen extract, the large nuclei were wrinkled and appeared half empty; some appeared pyknotic. Rarely was a mitotic figure observed and in these instances the chromosomes were clumped. A few small round tumor cells with small diploid nuclei having a fine regular chromatin reticulum resembled tumor cells from suspensions stored in 100 mgm. per ml. spleen extract at 5° C. for 2 days (Fig. 5). The cytological picture in general was one of nuclear degeneration and destruction of chromatin.

Nuclear volume. (Storage for 1, 2 and 5 days).—The average nuclear volume increased slightly during the first 2 days of storage in isotonic Ringer's solution over that of unstored nuclei. Of 50 nuclei measured at the end of 24 hours of storage, 48 per cent were over 1,200 cu. μ or of the larger Class III size of nucleus, according to the classification of Biesele, Poyner, and Painter (6); after 5 days, 40 per cent were of this type. The rest of the nuclei in both cases were under 1,200 cu. μ in volume, or Biesele's Class II diploid type. After 1 day in 60 mgm. per ml. spleen extract at 5° C., 40 per cent of the measured nuclei were of the Class III size, and after 5 days 23 per cent remained in this classification, indicating that the 60 mgm. per ml. spleen extract acting for 24 hours at 5° C. had had the same effect on nuclear volume as 5 days in isotonic Ringer's solution at 5° C. The average nuclear volume of 50 cells stored for 24 to 48 hours in 100 mgm. per ml. spleen extract in normal saline at 5° C. was less than that of control cells in isotonic Ringer's solution.

DISCUSSION

The observed effects of cold on dbrB tumor cells in isotonic Ringer's solution are in agreement with the report of Heilbrun (11) that cold prevented spindle formation in sea urchin eggs and that normal mitosis was resumed when the chilled cells became warm again. In contrast to the findings of Barber and Callan (3) that metaphase configurations increased 260 per cent in newt larvæ during an 8 day storage period at 3° C., we observed but few nuclei in metaphase during storage of dbrB tumor cell suspensions in isotonic Ringer's solution at 5° C. for as long as 7 days.

Many of the resting nuclei of the stored tumor cells resembled the non-proliferating malignant or Type B cells described by Koller (12, 13). The nucleoli of variable size in these non-proliferating cells consist of histone-protein and ribose nucleic acid with very little of the desoxyribose nucleic acid (12) which latter predominates in the small nucleoli of proliferating cells; these 2 nucleic acids are convertible one to the other in a reversible reaction in the normal mitotic cycle (8, 9). It is probable in our experiments that the resting type of nucleus in the tumor suspensions was reconverted into the proliferating type of nucleus upon injection into mice, since the takes were 100 per cent in nearly every control experiment. Among the smaller diploid nuclei in tumor cells stored in isotonic Ringer's solution for as long as 7 days, there were a few resembling those of the Type A proliferating cells described by Koller (12, 13) as having small Feulgen-positive nucleoli and conspicuous heterochromatic segments on the nuclear reticulum.

When dbrB tumor cells were stored in beef spleen extract at 5° C., the immediate elimination of almost all mitotic activity, the reduction of nucleolar size, and the presence of a coarse reticulum lacking darker staining segments, can be correlated with the number of tumors developing in the injected mice. When the tumor takes were 100 per cent with cells stored for 1 day in 60 mgm. per ml. spleen extract and for 4 days with 40 mgm. per ml. spleen extract, the nuclei were cytologically similar. When no tumors developed following injection of cells stored for 5 days with 60 mgm. per ml. spleen extract, the cytologic picture was one of destruction of nuclear material as manifested by empty and semi-empty nuclear membranes and by pyknosis; after 5 days of storage in isotonic Ringer's solution, tumor cell suspensions gave rise to tumors in 100 per cent of the animals although a few of the cells cytologically showed a decrease in chromatin content of the nuclei (Fig. 4). When tumors developed in all of the animals receiving

cell suspensions stored for 1 day at 5° C. in either hypertonic Ringer's solution or in 100 mgm. per ml. spleen extract, the chromatic reticulum in both instances was a fine regular network; the nucleoli persisted in those cells stored in Ringer's solution but had disappeared from most of the cells in the presence of the extract. Although there was little further cytologic change in these cells after 48 hours (Fig. 5), tumors developed in but 1 of 6 mice receiving suspensions stored in the hypertonic Ringer's solution, and in none of the 20 mice receiving those stored in 100 mgm. per ml. spleen extract.

A correlation of the cytologic characteristics of tumor cells stored in spleen extract at 5° C. with the results obtained *in vivo,* showed that the reduction in the number of tumors developing may be due to: (a) an immediate destruction of some cells; and (b) an accelerated degeneration of the nucleus with a deterioration of its contents.

The action of the spleen extract may be due in part to its hypertonicity since in its higher concentrations the large polyploid cells were damaged first and mitosis was inhibited almost immediately. Belar (4) observed that in living grasshopper spermatocytes exposed to hypertonic Ringer's solution, the cytoplasm was first affected, the spindle next, and finally the chromosomes. The prolonged latent period before tumors became palpable, in those instances when tumors did develop following exposure to spleen extract at 5° C., would seem to indicate that but few potentially dividing cells remained in the inoculum, rather than that the viability of each of the cells was reduced.

Although the nucleolus tended to persist in nuclei of cells in hypertonic Ringer's solution, and to disappear in those in spleen extract, a toxic effect of the extract on the malignant cells cannot be eliminated as a possible factor in the reduction of tumor takes from cells stored in spleen extract. Dustin (10) observed in the nuclei of intestinal epithelium of mice that *in vivo* the nucleolus was apparently more resistant than other cellular structures to poisons such as hydroquinone and the carbamates, which he classifies as radiomimetic poisons, and which seem to act on the enzyme systems. Nuclei of potentially dividing cells are most sensitive to the action of poisons just prior to prophase. The appearance of the nuclei as described by Dustin (10) during degeneration and pyknosis is similar to that of dbrB tumor cell nuclei after 5 days of storage with 60 mgm. per ml. spleen extract. Dustin (10) described the effects of another group of poisons, *i.e.*, mercurial compounds, arsenite and colchicine, on dividing cells *in vivo* as preventing spindle formation with a consequent accumulation of metaphase figures. This effect was not observed *in vitro* in dbrB tumor cells exposed to spleen extract, nor when 0.5 ml. of 260 mgm. per ml. spleen extract was injected into a small growing tumor 2 days before its excision; mitotic divisions were found with high frequency in these tumor cells. Neither resistance to nor regression of the dbrB tumors has been produced with the beef spleen extract. Cameron and her associates (7) have shown that the response of mouse tumor cells to organic poisons is not necessarily similar to that of human malignant cells.

Storage of mouse tumor cells in beef spleen extract at 5° C. has proved unsatisfactory as a method for assaying the potency of the spleen extract for clinical investigations.

SUMMARY

Injection of dbrB tumor cell suspensions after storage at 5° C. for 1 to 7 days in different concentrations of an aqueous extract of beef spleen resulted in a reduced number of tumors developing, this reduction being related to the dilution of the extract and the period of storage. In those instances in which tumors developed the latent period was prolonged over and above that for tumors from suspensions stored for the same period of time in isotonic Ringer's solution.

Cytologic examination revealed that in the presence of the spleen extract at 5° C. cell division disappeared, the nucleoli and heterochromatic segments were reduced in size, the number of vacuolated nuclei was increased, as well as the destruction of chromatic material leading to pyknosis and to hollow nuclear membranes.

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